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PATENT- OG VAREMÆRKESTYRELSEN

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SiLNA: RNAi augmenting by LNA

FIELD OF THE INVENTION

The present invention provides compositions and methods for double-stranded RNA (dsRNA) that induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). The dsRNA according to the present invention e.g. has enhanced properties through the utilisation of LNA (Locked Nucleic Acid).

BACKGROUND OF THE INVENTION

Discovery of RNA interference (RNAi) in C. Elegans was made by Fire *et al.* (Nature, 1998, 391, 806-811). Long stretches of double stranded RNA (dsRNA) had a potent knock-down effect that could last for generations in the worm. RNAi rapidly became a functional genomics tool in C. Elegans (Early RNA interference is reviewed by Fire, TIG, 1999,15, 358-363 and Bosher and Labouesse, Nature Cell Biology, 2000, 2, E31-E36). The first studies where RNA interference worked in vertebrates were performed in zebrafish embryos and mouse oocytes (Wargelius *et al.*, Biochem. Biophys. Res. Com. 1999, 263, 156-161, Wianny and Zernicka-Goetz, Nature Cell Biology, 2000, 2, 70-75). Since double stranded RNA induces non-specific effects in mammalian cells, (Alexopoulou *et al.*, Nature, 2001, 413, 732-738, Reviews: Stark *et al.*, Annu. Rev. Biochem., 1998, 67, 227-264 and Samuel, Clin. Micro. Rev., 2001, 14, 778-809) it was argued that these mechanisms were not fully developed in the mouse embryonic system.

In C. Elegans and Drosophila it was shown that the long RNAi strands were degraded to short double strands (21-23 nt) and that those were mediating the interference (Zamore *et al.*, Cell, 2000, 101, 25-33, Elbashir *et al* , Gen. Dev., 2001, 15, 188-200). Elbashir *et al.* (Gen. Dev., 2001, 15, 188-200) showed that a sense or antisense target is cleaved equally and that both strands in siRNA can guide cleavage to target antisense or sense RNA respectively. It was unambiguously shown by Elbashir *et al.* (Nature, 2001, 411, 494-

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498) that the short interfering RNAs (siRNAs) mediate potent knock-down in a variety of mammalian cell lines and probably escaped the adverse non-specific effects of long dsRNA in mammalian cells. This discovery was a hallmark in modern biology and the applications of RNAi became apparent also the future application of siRNA as therapeutics (Reviews: McManus and Sharp, Nature Reviews Genetics, 2002, 3, 737-747 and Thompson, DDT, 2002, 7, 912-917).

MODIFICATIONS OF siRNA DOUBLE STRANDS; RNAi duplexes are rather stable in biological media. But the moment the duplex is dissociated into the individual strands these are, by virtue of being RNA, immediately degraded. One of the strategies to bring further stability to siRNA has been to introduce chemically modified RNA residues (analogues) into the individual strands of the siRNA. It is well known that synthetic RNA analogues are much more stable in biological media, and that the increased stability is also induced to the proximate native RNA residues. By greater stability is mainly meant increased nuclease resistance but also better cellular uptake and tissue distribution is wanted. Several RNA analogues have been introduced into siRNA.

Pre-siRNA, Parrish *et al.* (Mol. Cell, 2000, 6, 1077-1087) show tolerance for certain backbone modifications for RNAi in C. elegans. They *in vitro* transcribed the two different strands in presence of modified nucleotides. They show that phosphorothioates are tolerated in both the sense and antisense strand and so are 2'-fluorouracil instead of uracil. 2'- Aminouracil and 2'-aminocytidine reduce the effect when incorporated into the sense strand and abolish completely effect in the antisense strand. With an exchange of uracil to 2'-deoxythymidine in the sense strand the effect is also reduced and even more when the exchange is in the antisense strand. Either strand consisting of all DNA abolishes the RNAi activity. They also performed base modifications. 4-Thiouracil and 5-bromouracil are tolerated in both stands. 5-Iodouracil and 5-(3-aminoallyl)uracil reduce the effect in the sense strand and even more in the antisense strand. Changing guanosine to inosine markedly reduce the effect whether present in the sense or antisense strand. However, UU 3'-overhangs can be exchanged with 2'deoxythymidine 3'-overhangs and are well tolerated (Elbashir *et al.*, Nature, 2001, 411, 494-498, Boutla *et al.*, Curr. Biol., 2001, 11, 1776-1780). It has also been shown that DNA can be incorporated in the sense

strand without compromising the effect. Elbashir *et al.*, EMBO, 2001, 20, 6877-6888) show in drosophila lysate that not only the 3'-overhang in siRNA can be exchanged for 2'-deoxynucleotides but two more, in total four deoxynucleotides in each 3'-end of the siRNA maintain full activity. All DNA as well as all 2'-O-methyl residues in either strand abolish the effect. They see abolished siRNA activity with only one base-pair mismatch in the middle. However, it has also been reported that one to two mis-matches can be tolerated as long as the mismatches are introduced in the sense strand (Holen *et al.*, NAR, 2002, 30, 1757-1766, Hohjoh, FEBS Lett., 2002, 26179, 1-5, Hamada *et al.*, Antisense and Nucl. Acid Drug Dev., 2002, 12, 301-309, Boutla *et al.*, Curr. Biol., 2001, 11, 1776-1780)).

Nykänen *et al.* (Cell, 2001, 107, 309-321) show the need for ATP in making siRNA out of RNAi, but also in the later steps to exert the siRNA activity. ATP is needed for unwinding and maintaining a 5'-phosphate for RISC recognition. The 5'-phosphate is necessary for siRNA activity. Martinez *et al.*(Cell, 2002, 110, 563-574) show that a single strand can reconstitute the RNA induced silencing complex (RISC, Hammond *et al* , Nature, 2000, 404, 293-296 and single antisense strand have activity especially when 5'-phosphorylated. 5'-Antisense strand modification inhibits activity while both 3'-end and 5'-sense end can be modified.

Amarzguioui *et al.* (NAR, 2003, 31, 589-595) have the same findings as above when it comes to mismatches, one is tolerated as long as it is not too close to the 5'-end of the antisense strand. A mismatch 3 and 5 nts from the 5'-end of the antisense strand markedly diminish the effect. However, they show that two mismatches are tolerated if they are in the middle or towards the 3'-end of the antisense strand, though with a slightly reduced effect. Mismatches close to the 5'-end of the antisense strand abolish the effect. Single stranded antisense RNA have in their system an inhibitory effect close to the one of double stranded siRNA. Where as the end modified antisense RNA show less effect compared to corresponding siRNA. Phosphorylation of the 5'-antisense end does not enhance the efficacy.

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Modifications such as phosphorothioates and 2'-O-methyl RNA have been introduced at the termini of siRNA (Amarzguioui *et al.*, NAR, 2003, 31, 589-595) and they were tolerated. 2'-O-allylation reduces the effect when present in the 5'-end of the antisense strand. The effect of the modified siRNA is stronger than unmodified siRNA after five days.

The bi-cyclic nucleoside analogue ENA (2'-O,4'-C-ethylene thymidine (ENA thymidine, eT) has also been incorporated into siRNA (Hamada *et al.*, Antisense and Nucl. Acid Drug Dev., 2002, 12, 301-309). It was shown that even minimally modification of siRNA, two eT in the 5'-end of the sense strand, deteriorated the effect. It was concluded by Hamada *et al.* (2002) that: "using 2'-*O*,4'-*C*-ethylene thymidine, which is a component of ethylene-bridged nucleic acids (ENA), completely abolished RNAi".

In conclusion the antisense strand is much more sensitive to modifications than the sense strand. This is at least partly based on the fact that the structure of the antisense/target duplex has to be native A-form RNA. The sense strand of siRNA can be regarded as a "vehicle" for the delivery of the antisense strand to the target and it is not participating in the enzyme-catalyzed degradation of RNA. In contrast to the antisense strand modifications of the sense strand is tolerated with-in a certain window even though the modifications induce changes to the A-form structure of the siRNA. If changes are introduced in the antisense strand they have to be structurally balanced with-in the recognition frame of the native RNA induced silencing complex (RISC).

SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that LNA (Locked Nucleic Acid) can be used to improve RNAi on important parameters. This is a surprising finding due to the fact that the structural closely related ENA completely deteriorates the effect of even minimally modified siRNA duplexes.

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The present invention is directed to siRNA-like compounds comprising at least one LNA monomer that enable gene silencing via a RNAi mechanism. These novel compounds are defined as "siLNA". Pharmaceutical and other compositions comprising these compounds of the invention are also provided. Further provided are methods of oligonucleotide preparation and gene silencing of a target in cells or tissues comprising contacting said cells or tissues with one or more of the siLNA compositions of the invention. Also disclosed are methods of treating an animal or a human, suspected of having or being prone to a disease or condition, associated with target gene by administering a therapeutically or prophylactically effective amount of one or more of the siLNA compositions of the invention. Further, methods of using siLNA of the inhibition of expression of target and for treatment of diseases associated with these targets are provided. Examples of such diseases are different types of cancer, such as for instance lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries.

A specific embodiment of the present invention is siLNA compounds targeting to SARS.

BRIEF DESCRIPTION OF THE FIGURES

Table 1. Design and Sequences of the LNA oligonucleotides used in siLNA experiments.

Figure 1. Furanose conformations.

Figure 2. Improved stability by siLNA over siRNA. GL3+/-, unmodified siRNA, is rapidly degraded while lightly modified siLNA (2185/2186) and more heavily modified siLNA (2703-01/2186) have markedly improved stability. In the siRNA case an accumulation of an intermediate band can be seen (inbetween dsRNA and ssRNA) that has been identified to be a doublestranded 19-mer, ie siRNA with degraded 3' overhangs. This is almost basically not seen when using siLNA. The degradation was performed in 10% foetal bovine serum in physiological salt solution at 37°C. Band was quantified and plotted in a graph.

Figure 3 SiRNA Inhibition of endogenous target by siLNA oligonucleotides

Figure 4 siLNA targeting firefly luciferase and modulation of the expression. The lines (green) represent the sense (left) and antisense (right) strand in the siLNA. The marks

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(red) represent the position of the LNA monomers. Grey lines, last bar on the left, are control siRNA. First bar represent full, non-modulated luciferase reporter expression, to which all samples are normalized.

Figure 5. siLNA targeting *Renilla* luciferase and modulating the expression. The lines (green) represent the sense (left) and antisense (right) strand in siLNA. The marks (red) represent the position of the LNA monomers. First bar represent full, non-modulated luciferase reporter expression, to which all samples are normalized.

Figure 6. Stability of LNA containing RNA monomers, double stranded RNA and single stranded in RAT serum. Double stranded (ds) RNA and single stranded (ss) RNA are immediately degraded while intact LNA containing RNA monomers can be detected up to 20 to 40 minutes.

Figure 7 SiLNA and siRNA compounds according to the invention targeting SARS.

Figure 8 Cytopathic effect (CPE) of vero cells when infected with SARS and reduced CPE by siRNA treatment. Shown is siRNA SARS 1. Mock is treated with the transfectionagent lipofectamine 2000 alone. Also shown is uninfected cells.

Figure 9 Inhibition of SARS induced cytotoxicity by siRNA and siLNA. The most efficient site SARS 1 is equally good with siLNA and siRNA. The medium efficient siRNA site SARS 3 is improved by siLNA to be as efficient as the SARS 1. The two sites that have not shown siRNA efficiency at all, SARS 2 and SARS 4, do not show any effect by siLNA either. The inhibitory effect is reduced at high viral doses (ie 60000 TCID50). Irrelevant controls are luciferase (Luc) and neuropeptide Y (NPY). No adverse effects are seen by the siLNA controls. Cytotoxicity is measured as lactate dehydrogenase (LDH) release at 50 hours post infection. The different graphs represent different viral doses (tissue culture infectious dose 50, TCID50). Data are shown as mean and standard deviation of three separate experiments in quadruplicates.

Figure 10 Mechanism of action

DEFINITIONS

The term "**LNA**" comprises an oligonucleotide comprising at least one locked nucleotide shown in scheme 2. One example of such a LNA monomeric residue comprising

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Thymine as nucleobase is: (1S,3R,4R, 7S)-7-hydroxy-1-hydroxymethyl-3-(thymin-1yl)-2,5-dioxa-bicyclo[2:2:1]heptane.

The term "**LNA monomer**" is equivalent to structures shown in Scheme 2.

The term "**siLNA**" is understood as a duplex of RNA/DNA/nucleic acid analogues active in RNAi in which at least one LNA monomer is incorporated.

As used herein, the terms "**siRNA**" refers to a compound, e.g. 21 nucleotides long double stranded stretch of RNA or modified RNA. The two strands in the siRNA usually have 19 nucleotides complementary to each other creating a double strand that is 19 nucleotides long and each strand having a 3'-end of two overhanging nucleotides. This is not a strict definition and siRNA that could be slightly longer or shorter with or without overhangs. In siRNA one strand is guiding to the target, and one strand is complementary to the target RNA and is referred to as the antisense strand. The other strand is referred to as the sense strand.

As used herein, the term "**mRNA**" means the presently known mRNA transcript(s) of a targeted gene, and any further transcripts, which may be identified.

As used herein, the terms "**target nucleic acid**" encompass any RNA that would be subject to modulation, targeted cleavage, steric blockage (decrease the abundance of the target RNA and/or inhibit translation) guided by the antisense strand (complementary to the target RNA, nucleic acid) in the siRNA. The target RNA could fro example be genomic RNA, genomic viral RNA, mRNA or a pre-mRNA

As used herein, the terms "**target-specific nucleic acid modification**" means any modification to target nucleic acid.

As used herein, the term "**gene**" means the gene including exons, introns, non-coding 5'- and 3'-regions and regulatory elements and all currently known variants thereof and any further variants, which may be elucidated.

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As used herein, the term "**modulation**" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

As used herein, the term "**targeting**" an siLNA or siRNA compound to a particular target nucleic acid means providing the siRNA oligonucleotide to the cell, animal or human in such a way that the siLNA or siRNA compound are able to bind to and modulate the function of its intended target.

As used herein, "**hybridisation**" means hydrogen bonding, which may be Watson-Crick, Hoogsteen, reversed Hoogsteen hydrogen bonding, etc. between complementary nucleoside or nucleotide bases. Watson and Crick showed fifty years ago that deoxyribo nucleic acid (DNA) is composed of two strands which are held together in a helical configuration by hydrogen bonds formed between opposing complementary nucleobases in the two strands. The four nucleobases, commonly found in DNA are guanine (G), adenine (A), thymine (T) and cytosine (C) of which the G nucleobase pairs with C, and the A nucleobase pairs with T. In RNA the nucleobase thymine is replaced by the nucleobase uracil (U), which similarly to the T nucleobase pairs with A. The chemical groups in the nucleobases that participate in standard duplex formation constitute the Watson-Crick face. Hoogsteen showed a couple of years later that the purine nucleobases (G and A) in addition to their Watson-Crick face have a Hoogsteen face that can be recognised from the outside of a duplex, and used to bind pyrimidine oligonucleotides via hydrogen bonding, thereby forming a triple helix structure.

In the context of the present invention "**complementary**" refers to the capacity for precise pairing between two nucleotides or nucleoside sequences with one another. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the corresponding position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to

each other at that position. The DNA or RNA and the oligonucleotide are considered complementary to each other when a sufficient number of nucleotides in the oligonucleotide can form hydrogen bonds with corresponding nucleotides in the target DNA or RNA to enable the formation of a stable complex. To be stable *in vitro* or *in vivo* the sequence of a siLNA or siRNA compound need not be 100% complementary to its target nucleic acid. The terms "complementary" and "specifically hybridisable" thus imply that the siLNA or siRNA compound binds sufficiently strongly and specifically to the target molecule to provide the desired interference with the normal function of the target whilst leaving the function of non-target mRNAs unaffected

The term **"Nucleic Acid Analogues"** refers to a non-natural nucleic acid binding compound. Nucleic Acid Analogues are described in e.g. Freier & Altmann (Nucl. Acid Res., 1997, 25, 4429-4443) and Uhlmann (Curr. Opinion in Drug & Development (2000, 3(2): 293-213). Scheme 1 illustrates selected examples.

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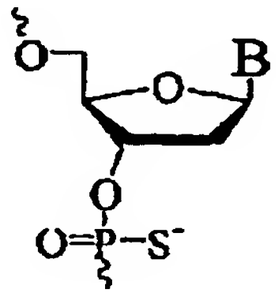
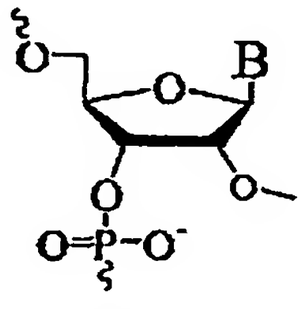


Phosphorthioate



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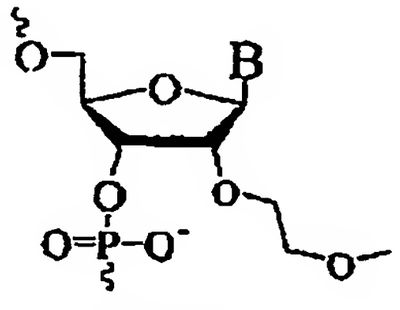

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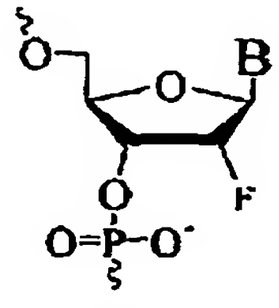
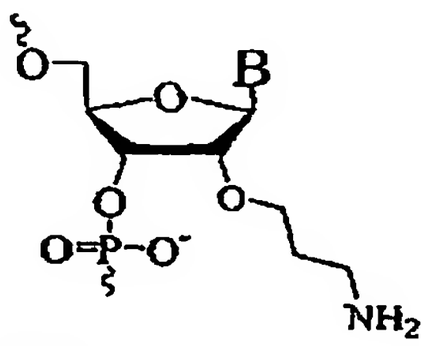
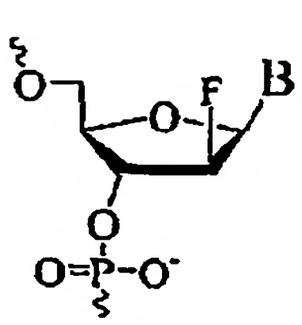


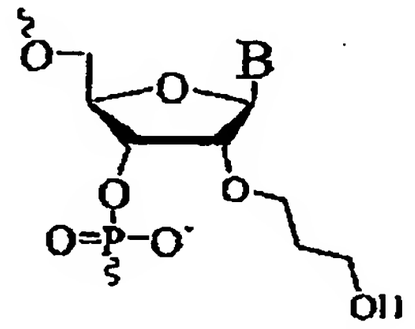
2'-Fluoro



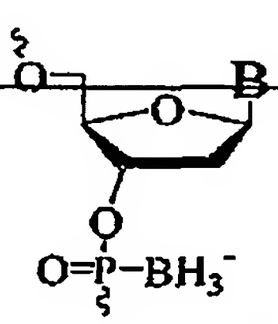
2'-AP



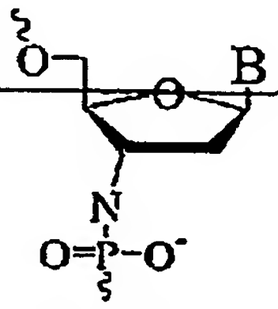
2'-F-ANA



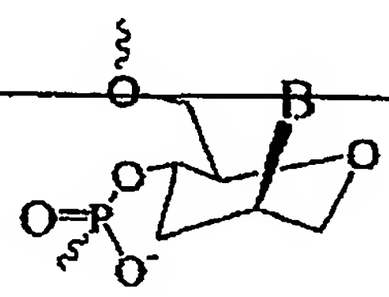
2'-(3-hydroxy)propyl



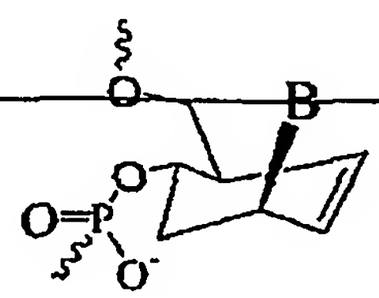
Boranophosphates



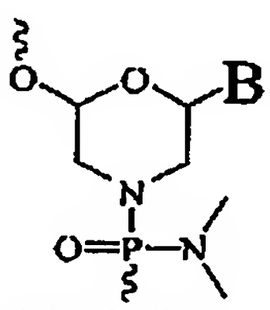
3'-Phosphoramidate



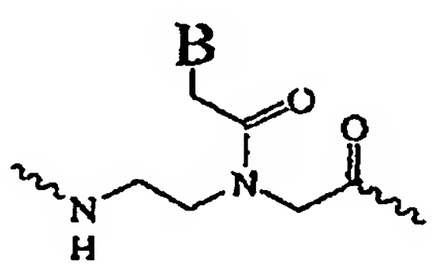
HNA



CeNA



Morpholino



PNA

Scheme 1

By the term "**inter nucleoside linkage**" is understood a chemical group that forms the linkage between natural or non-natural nucleosides, e.g. phosphatediesters and phosphorthioates.

The term "**oligonucleotide**" refers, in the context of the present invention, to an oligomer (also called oligo) or nucleic acid polymer (e.g. ribonucleic acid (RNA) or deoxyribonucleic acid (DNA)) or nucleic acid analogue of those known in the art, or LNA, or combinations thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring residues which function similarly or with specific improved functions. Fully or partly modified or substituted oligonucleotides are often preferred over native forms because of several desirable properties of such oligonucleotides such as for instance, the ability to penetrate a cell membrane, good resistance to extra- and intracellular nucleases, high affinity and specificity for the nucleic acid target. LNA exhibiting the above-mentioned properties is particularly preferred.

By the term "**improved properties**" is understood one or more parameters by which the siLNA comprised in this invention show better overall performance compared to their native counterparts. Examples of such parameters are ease of production, cost of production, longer shelf life of drug, higher binding constant to target (interim complement in siLNA or mRNA target), higher ability to penetrate a cell membrane, good resistance to extra- and intracellular nucleases, easier to formulate pharmaceutically, higher potency in mode of action, better tissue distribution, better phenotypic response, longer lasting effects.

The term "**at least one**" comprises the integers larger than or equal to 1, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and so forth.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to siRNA like compounds comprising at least one LNA monomer, enabling gene silencing, RNAi. These novel compounds are defined as "siLNA".

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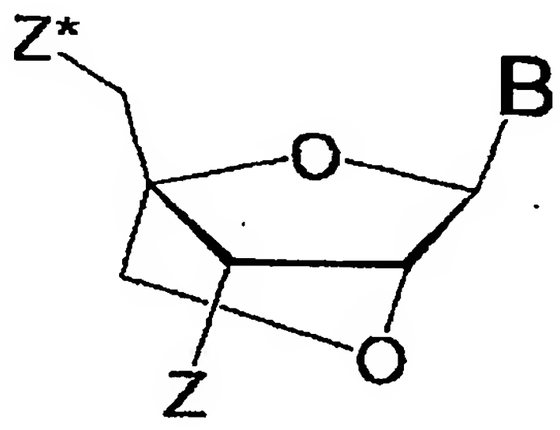
Also provided in this invention are pharmaceutical and other compositions comprising the siLNA compounds. Further provided are methods of gene silencing of a target in cells or tissues comprising contacting said cells or tissues with one or more of the siLNA compounds or compositions of the invention. Also disclosed are methods of treating an animal or a human, suspected of having or being prone to a disease or condition, associated with target gene by administering a therapeutically or prophylactically effective amount of one or more of the siLNA compounds or compositions of the invention. Further, methods of using siLNA compounds for the inhibition of expression of target and for treatment of diseases associated with these target are provided. Examples of such diseases are different types of cancer, such as for instance lung, breast, colon, prostate, pancreas, lung, liver, thyroid, kidney, brain, testes, stomach, intestine, bowel, spinal cord, sinuses, bladder, urinary tract or ovaries.

LNA monomers refers to a bicyclic nucleoside compound shown in Scheme 2



Scheme2

Z and Z* are independently selected among an internucleoside linkage, a terminal group or a protecting group.

The internucleoside linkage may be -O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -S-P(O)$_2$-O-, -S-P(O,S)-O-, -S-P(S)$_2$-O-, -O-P(O)$_2$-S-, -O-P(O,S)-S-, -S-P(O)$_2$-S-, -O-PO(R^H)-O-, O-PO(OCH$_3$)-O-, -O-PO(NR^H)-O-, -O-PO(OCH$_2$CH$_2$S-R)-O-, -O-PO(BH$_3$)-O-, -O-PO(NHR^H)-O-, -O-P(O)$_2$-NR^H-, -NR^H-P(O)$_2$-O-, -NR^H-CO-O-, -NR^H-CO-NR^H-, -O-CO-O-, -O-CO-NR^H-, -NR^H-CO-CH$_2$-, -O-CH$_2$-CO-NR^H-, -O-CH$_2$-CH$_2$-NR^H-, -CO-NR^H-

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CH_2-, -CH_2-NR^H-CO-, -O-CH_2-CH_2-S-, -S-CH_2-CH_2-O-, -S-CH_2-CH_2-S-, -CH_2-SO_2-CH_2-, -CH_2-CO-NR^H-, -O-CH_2-CH_2-NR^H-CO -, –CH_2-NCH_3-O-CH_2-, where R^H is selected form hydrogen and C_{1-4}-alkyl,

The terminal groups are selected independently among from hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, C_{1-6}-alkylthio, amino, Prot-N(R^H)-, Act-N(R^H)-, mono- or di(C_{1-6}-alkyl)amino, optionally substituted C_{1-6}-alkoxy, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted C_{2-6}-alkenyloxy, optionally substituted C_{2-6}-alkynyl, optionally substituted C_{2-6}-alkynyloxy, monophosphate-or protected monophosphate, monothiophosphate-or protected monothiophosphate, diphosphate-or protected diphosphate, dithiophosphate – or protected dithiophosphate, triphosphate- or protected triphosphate, trithiophosphate – or protected trithiophosphate. Examples of such protection groups on the phosphate residues are S-acetylthioethyl (SATE) or S-pivaloylthioethyl (*t*-butyl-SATE).

Also comprised as terminal groups are DNA intercalators, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, ligands, carboxy, sulphono, hydroxymethyl, Prot-O-CH_2-, Act-O-CH_2-, aminomethyl, Prot-N(R^H)-CH_2-, Act-N(R^H)-CH_2-, carboxymethyl, sulphonomethyl, where Prot is a protection group for -OH, -SH, and -NH(R^H), respectively, Act is an activation group for -OH, -SH, and -NH(R^H), respectively, and R^H is selected from hydrogen and C_{1-6}-alkyl.

The protection groups of hydroxy substituents comprises substituted trityl, such as 4,4'-dimethoxytrityloxy (DMT), 4-monomethoxytrityloxy (MMT), and trityloxy, optionally substituted 9-(9-phenyl)xanthenyloxy (pixyl), optionally substituted methoxytetrahydropyranyloxy (mthp), silyloxy such as trimethylsilyloxy (TMS), triisopropylsilyloxy (TIPS), *tert*-butyldimethylsilyloxy (TBDMS), triethylsilyloxy, and phenyldimethylsilyloxy, *tert*-butylethers, acetals (including two hydroxy groups), acyloxy such as acetyl or halogen substituted acetyls, *e.g.* chloroacetyloxy or fluoroacetyloxy, isobutyryloxy, pivaloyloxy, benzoyloxy and substituted benzoyls, methoxymethyloxy (MOM), benzyl ethers or substituted benzyl ethers such as 2,6-dichlorobenzyloxy (2,6-Cl_2Bzl).

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Alternatively when Z or Z* is hydroxyl they may be protected by attachment to a solid support optionally through a linker.

When Z or Z* is amino groups illustrative examples of the amino protection protections are fluorenylmethoxycarbonylamino (Fmoc), *tert*-butyloxycarbonylamino (BOC), trifluoroacetylamino, allyloxycarbonylamino (alloc, AOC), Z benzyloxycarbonylamino (Cbz), substituted benzyloxycarbonylaminos such as 2-chloro benzyloxycarbonylamino (2-ClZ), monomethoxytritylamino (MMT), dimethoxytritylamino (DMT), phthaloylamino, and 9-(9-phenyl)xanthenylamino (pixyl).

In the embodiment above, Act designates an activation group for -OH, -SH, and -$NH(R^H)$. In a preferred embodiment such activators mediates couplings to other residues, monomers. After such successful couplings the act-group is converted to an internucleoside linkage. Such activation groups are, *e.g.*, selected from optionally substituted O-phosphoramidite, optionally substituted O-phosphortriester, optionally substituted O-phosphordiester, optionally substituted H-phosphonate, and optionally substituted O-phosphonate.

In the present context, the term "**phosphoramidite**" means a group of the formula $-P(OR^x)-N(R^y)_2$, wherein R^x designates an optionally substituted alkyl group, *e.g.* methyl, 2-cyanoethyl, or benzyl, and each of R^y designate optionally substituted alkyl groups, *e.g.* ethyl or isopropyl, or the group $-N(R^y)_2$ forms a morpholino group ($-N(CH_2CH_2)_2O$). R^x preferably designates 2-cyanoethyl and the two R^y are preferably identical and designate isopropyl. Thus, an especially relevant phosphoramidite is N,N-diisopropyl-*O*-(2-cyanoethyl)phosphoramidite.

B constitutes a natural or non-natural nucleobase and selected among adenine, cytosine, 5-methylcytosine, isocytosine, pseudoisocytosine, guanine, thymine, uracil, 5-bromouracil, 5-propynyluracil, 5-propyny-6-fluoroluracil, 5-methylthiazolcuracil, 6-aminopurine, 2-aminopurine, inosine, 2,6-diaminopurine, 7-propyne-7-deazaadenine, 7-propyne-7-deazaguanine, 2-chloro-6-aminopurine.

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Particularly preferred bicyclic structures are structures in which Z and Z* are independently selected among an internucleoside linkage comprizing- O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -S-P(O)$_2$-O-, -S-P(O,S)-O-, -S-P(S)$_2$-O-, -O-P(O)$_2$-S-, -O-P(O,S)-S-, -S-P(O)$_2$-S-, -O-PO(R^H)-O-, O-PO(OCH$_3$)-O-, -O-PO(NR^H)-O-, -O-PO(OCH$_2$CH$_2$S-R)-O-, -O-PO(BH$_3$)-O-, -O-PO(NHR^H)-O-, -O-P(O)$_2$-NR^H-, -NR^H-P(O)$_2$-O-, -NR^H-CO-O-, where R^H is selected form hydrogen and C$_{1-4}$-alkyl,

a terminal group comprising hydrogen, azido, halogen, cyano, nitro, hydroxy, Prot-O-, Act-O-, mercapto, Prot-S-, Act-S-, C$_{1-6}$-alkylthio, amino, Prot-N(R^H)-, Act-N(R^H)-, mono- or di(C$_{1-6}$-alkyl)amino, optionally substituted C$_{1-6}$-alkoxy, optionally substituted C$_{1-6}$-alkyl, optionally substituted monophosphate, monothiophosphate, diphosphate, dithiophosphate triphosphate, trithiophosphate, where Prot is a protection group for -OH, -SH, and -NH(R^H), respectively, Act is an activation group for -OH, -SH, and -NH(R^H), respectively, and R^H is selected from hydrogen and C$_{1-6}$-alkyl,

a protecting group of hydroxy substituents comprizing substituted trityl, such as 4,4'-dimethoxytrityloxy (DMT), 4-monomethoxytrityloxy (MMT), optionally substituted 9-(9-phenyl)xanthenyloxy (pixyl), optionally substituted methoxytetrahydropyranyloxy (mthp), silyloxy such as trimethylsilyloxy (TMS), triisopropylsilyloxy (TIPS), *tert*-butyl-dimethylsilyloxy (TBDMS), triethylsilyloxy, and phenyldimethylsilyloxy, *tert*-butylethers, acetals (including two hydroxy groups), acyloxy such as acetyl Alternatively when Z or Z* is hydroxyl they may be protected by attachment to a solid support optionally through a linker.

When Z or Z* is amino groups illustrative examples of the amino protection protections are fluorenylmethoxycarbonylamino (Fmoc), *tert*-butyloxycarbonylamino (BOC), trifluoroacetylamino, allyloxycarbonylamino (alloc, AOC), monomethoxytritylamino (MMT), dimethoxytritylamino (DMT), phthaloylamino.

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In the embodiment above, Act designates an activation group for -OH, -SH, and -H(R^H)-, respectively. Such activation groups are, *e.g.*, selected from optionally substituted O-phosphoramidite, optionally substituted O-phosphortriester, optionally substituted O-phosphordiester, optionally substituted H-phosphonate, and optionally substituted O-phosphonate.

In the present context, the term "**phosphoramidite**" means a group of the formula -P(OR^x)-N(R^y)$_2$, wherein R^x designates an optionally substituted alkyl group, *e.g.* methyl, 2-cyanoethyl, and each of R^y designate optionally substituted alkyl groups, R^x preferably designates 2-cyanoethyl and the two R^y are preferably identical and designate isopropyl. Thus, an especially relevant phosphoramidite is N,N-diisopropyl-*O*-(2-cyanoethyl)-phosphoramidite.

B constitutes a natural or non-natural nucleobase and selected among adenine, cytosine, 5-methylcytosine, isocytosine, pseudoisocytosine, guanine, thymine, uracil, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine, 2-chloro-6-aminopurine.

Therapeutic principle

In one embodiment of the invention the siLNA compounds are suitable drugs. The design of a potent and safe RNAi drug requires the fine-tuning of diverse parameters such as affinity/specificity, stability in biological fluids, cellular uptake, mode of action, pharmacokinetic properties and toxicity.

Affinity & specificity

LNA exhibits unprecedented binding properties towards DNA and RNA target sequences. In addition to these remarkable hybridization properties, LNA monomers can be mixed and act cooperatively with DNA and RNA monomers, and with other nucleic acid analogues, such as 2'-O-alkyl modified RNA monomers. As such, the oligonucleotides of the present invention can be composed entirely of LNA monomers or it may be composed of LNA monomers in any combination with DNA, RNA or

contemporary nucleic acid analogues. The unprecedented binding affinity of LNA towards DNA or RNA target sequences, and the ability to mix LNA monomers freely with DNA, RNA monomers and a range of contemporary nucleic acid analogues has a range of important consequences according to the invention for the development of effective and safe RNAi compounds.

Natural dsDNA exists at physiological pH as a B-form helix, whereas dsRNA exists as an A-form helix. This morphological difference is originated in the difference in the preferred sugar conformations of the deoxyriboses and the riboses. The furanose ring of deoxyribose exists at room temperature in an equilibrium between C2'-*endo* (*S*-type) and C3'-*endo* (*N*-type) conformation with an energy barrier of ~2 kcal/mol (Figure 1). The C2'-*endo* (*S*-type) conformation gives rise to the B-form helix whereas the C3'-*endo* (*N*-type) conformation gives rise to the A-form helix.

For deoxyribose the S-type conformation is slightly lowered in energy (~0.6 kcal/mol) compared to the N-type and explains why DNA is found in the S-type conformation. For ribose the preference is for the N-type and thus, RNA adopts the A-form helix. The A-form helix is associated with higher hybridisation stability.

LNA monomers are locking the conformation of the furanose in a representation that corresponds to an extreme 3'-endo conformation. These monomers are therefore mimicking the RNA conformation, and it has been shown that the structure of the oligonucleotide and duplexes of the monomers are RNA like (Petersen et al., J. Am. Chem. Soc., 2002, 124, 5974-82). This means that the structure of the RNA oligonucleotides and the RNA/RNA duplexes in which the LNA monomers are incorporated into are not significantly changed compared to native RNA oligonucleotides and RNA/RNA duplexes. It was furthermore shown that the LNA monomers induced RNA-like conformation in DNA. Thus, the LNA monomers imposed on the DNA residues, in particular to the 3'end, a strong degree of C3'-endo conformation (RNA like). If for instance every second or third residue in a DNA oligomer is replaced with LNA monomers the overall structure of the oligonucleotide will become much like RNA. Thus, the duplex formed by such oligonucleotides will attain a structure resembling

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native A-form duplexes (RNA/RNA). It is part of this invention to use this property of the LNA monomers to direct the conformation of DNA towards RNA structure, and thereby interchange RNA monomers with DNA monomers without disturbing the RNA-like structure.

The siLNA according to the present invention is preferably 12-35, more preferably 17-25 and most preferably 20-21 nucleotides in length.

The siLNA according to the present invention may be blunt ended. The siLNAs may also have a 3' overhang, at least on one strand, of 1-7 nucleotides, preferably 1-3 nucleotides. The siLNAs may also have a 5' overhang, at least on one strand, of 1-4 nucleotides, preferably 1-3 nucleotides.

The siLNA according to the present invention has an antisense sequence, which has least 70 percent, preferably 90-100% sequence identity to the target molecule.

In one embodiment of the invention it is the intention to use the unprecedented affinity of the LNA to shortening the usual length of a siRNA oligonucleotide (from 20-25 mers to, *e.g.*, 12-15 mers) without compromising the affinity required for pharmacological activity. As the *intrinsic specificity* of an oligonucleotide is inversely correlated to its length, such a shortening will significantly increase the specificity of the siLNA compound towards its RNA target. One embodiment of the invention is to, due to the fact that the sequence of the humane genome is available and the annotation of its genes rapidly progressing, identify the shortest possible, unique sequences in the target mRNA.

In another embodiment is to use LNA's properties to reduce the size of oligonucleotides significantly and thereby ease the process - and price of manufacture thus providing the basis for RNAi therapy to become a commercially competitive treatment offer for a diversity of diseases.

In another embodiment, the unprecedented affinity of LNA can be used to substantially enhance the ability of an RNAi oligonucleotide to hybridize to its target mRNA *in-vivo*

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thus significantly reducing the time and effort required for identifying an active compound as compared to the situation with other chemistries.

In another embodiment, the unprecedented affinity of LNA is used to enhance the potency of siRNA oligonucleotides thus enabling the development of compounds with more favorable therapeutic windows than those currently in clinical trials.

When designed as a siRNA inhibitor, the oligonucleotides of the invention bind to the target nucleic acid and modulate the expression of its cognate protein. Preferably, such modulation produces an inhibition of expression of at least 10% or 20% compared to the normal expression level, more preferably at least a 30%, 40%, 50%, 60%, 70%, 80%, or 90% inhibition compared to the normal expression level.

Typically, the siLNA of the invention will contain other residues than LNA monomers such as native RNA monomers, DNA monomers, N3'-P5'phosphoroamidates, 2'-F, 2'-O-Me, 2'-O-methoxyethyl (MOE), 2'-O-(3-aminopropyl) (AP), hexitol nucleic acid (HNA), 2'-F-arabino nucleic acid (2'-F-ANA) and D-cyclohexenyl nucleoside (CeNA). In general, the individual strands of the siLNA will contain at least about 5, 10, 15 or 20 percent LNA monomer, based on total nucleotides of the oligonucleotide, more typically at least about 20, 25, 30, 40, 50, 60, 70, 80 or 90 percent LNA monomer based on total bases of the oligonucleotide.

In a specific embodiment of the present invention the siLNA compounds is a 19-mer and blunt ended.

A specific embodiment of the present invention is siLNA compounds targeting to Severe Acute Respiratory Syndrome (SARS), which first appeared in China in November 2002. Since then, over 8,000 people have been infected world-wide resulting in over 900 deaths according to the WHO. A previously unknown coronavirus has been identified as the causative agent for the SARS epidemic (Drosten C, Gunther S, Preiser W, et al. N Engl J Med 2003; 348:1967-76; Fouchier RA, Kuiken T, Schutten M, et al. Nature 2003;

423:240). Identification of the SARS-CoV was followed by rapid sequencing of the viral genome of multiple isolates (Ruan Y, C. W, A. E, et al. Lancet 2003; 361:1779-85; Rota PA, Oberste MS, Monroe SS, et al. Science 2003; 300:1394-9; Marra MA, Jones SJ, Astell CR, et al. Science 2003; 300:1399-404). This sequence information immediately made possible the development of SARS antivirals by nucleic acid based knock-down techniques such as siRNA. The nucleotide sequence encoding the SARS-CoV RNA dependent RNA polymerase (Pol) is highly conserved throughout the coronavirus family. The Pol gene product is translated from the genomic RNA as a part of a polyprotein, and uses the genomic RNA as a template to synthesize negative-stranded RNA and subsequently sub-genomic mRNA. The Pol protein is thus expressed early in the viral life cycle and is crucial to viral replication (see Figure 10).

Stability in biological fluids

One embodiment of the invention includes the incorporation of LNA monomers into a standard DNA or RNA oligonucleotide to increase the stability of the resulting siLNA compound in biological fluids e.g. through the increase of resistance towards nucleases (endonucleases and exonucleases). The extent of stability will depend on the number of LNA monomers used, their position in the oligonucleotides and the type of LNA monomer used. Compared to DNA and phosphorothioates the following order of ability to stabilize an oligonucleotide against nucleolytic degradation can be established: DNA << phosphorothioates, LNA-phosphordiester < LNA- phosphorothioates.

Given the fact that LNA synthesis is compatible with standard RNA/DNA synthesis and that the LNA monomers mixes freely with many contemporary nucleic acid analogues, nuclease resistance of siLNA compounds can be further enhanced according to the invention by either incorporating other analogues that display increased nuclease stability or by exploiting nuclease-resistant internucleoside linkages e.g. phosphoromonothioate, phosphorodithioate, and methylphosphonate linkages, etc.

siRNA DESIGNS

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LNA monomers can be used freely in the design of siLNA at both 3'-overhangs and the 5'-end of the sense strand with full activation of the siLNA effect, down regulation of protein production (>90% reduction). LNA monomers can be distributed quite freely over the sense strand in the siLNA with maintained high down regulating capability (80% reduction). The 5'-end of the antisense strand in the siLNA can also be modified by LNA monomers and giving down regulatory capabilities of up to 50-70 %. Using a highly LNA monomer substituted antisense strand has not given a down regulatory effect, although it can not be excluded that special design of that combination can elicit RNAi effects. LNA monomer substitutions of the 3'-overhangs along with the 5'-end of the sense strand of the siLNA give the highest reductions of protein levels. 5'-end of the antisense strand is the most sensitive to the LNA unit modification while many other sites of modification are better tolerated.

One embodiment of the invention relates to the siLNA compounds and designs shown in table 1 or 2 or Figure 2.

In one embodiment of the invention at least on LNA monomer is incorporated in any positions except the 5'-end of the antisense strand.

In one embodiment the siLNA compound is designed so that the monomers are LNA incoporeted in the compound in such a way they are strengthening the basepairs in the 5'-end of the sense/complementery strand. The helicase can be directed to unwinde from the other 5'-end. In this way the incorporation of the antisense/guiding strand into RISC can be controlled. The helicase starts unwinding the siRNA duplex the weakest binding end. The released 3' end is probably targeted for degradation while the remaining strand is incorporated in the RISC. Efficient siRNA show accumulation of the antisense/guiding strand and weaker base pairing in the 5'-end of the antisense/guiding strand. Possibly could also unwanted side effects be avoided by having only the correct strand (the antisense/complementary) in RISC and not the unwanted sense/complementary strand.

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PHARMACOKINETIC PROPERTIES

The clinical effectiveness of siRNA or siLNA oligonucleotides depends to a significant extent on their pharmacokinetics e.g. absorption, distribution, cellular uptake, metabolism and excretion. In turn these parameters are guided significantly by the underlying chemistry and the size and three-dimensional structure of the oligonucleotide.

As mentioned earlier siLNA according to the invention is not related to a single, but several related chemistries, which although molecularly different all exhibit stunning affinity towards complementary DNA and RNA. Thus, the design of the individual strands in siLNA may contain other residue than LNA monomers. Above is mentioned that RNA monomers will be usual part of the oligonucleotides but also DNA monomers can be present. Also nucleic acid analogues (scheme 1) can play part of the oligonucleotides. Other possible compositions are mixed backbones (described above), e.g. a combination of phosphordiester and phosphorthioates, or fully modified phosphorthioate backbones.

In the latter case, the use of for instance a fully modified phosphorthioate backboneswill change the overall lipophilicity of the oligonucleotide and affect uptake and distribution behavior, and thus influence its ability to pass through lipophilic barriers such as for instance the cell membrane.

Such modulating the pharmacokinetic properties of a siLNA according to the invention may further be achieved through attachment of a variety of different moieties. For instance, the ability of oligonucleotides to pass the cell membrane may be enhanced by attaching for instance lipid moieties such as a cholesterol moiety, a thioether, an aliphatic chain, a phospholipid or a polyamine to the oligonucleotide. Likewise, uptake of siLNA into cells may be enhanced by conjugating moieties to the oligonucleotide that interacts with molecules in the membrane, which mediates transport into the cytoplasm.

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PHARMACODYNAMIC PROPERTIES

The pharmacodynamic properties can according to the invention be enhanced with groups that improve oligonucleotide uptake, enhance biostability such as enhance oligonucleotide resistance to degradation, and/or increase the specificity and affinity of oligonucleotides hybridisation characteristics with target sequence e.g. a mRNA sequence.

TOXICOLOGY

There are basically two types of toxicity associated with siLNA oligonucleotides: sequence-dependant toxicity, involving the base sequence, and sequence-independent, class-related toxicity. With the exception of the issues related to immunostimulation by native CpG sequence motifs, the toxicities that have been the most prominent in the development of oligonucleotide drugs are independent of the sequence, e.g. related to the chemistry of the oligonucleotide and dose, mode, frequency and duration of administration. The phosphorothioate class of oligonucleotides have been particularly well characterized and found to elicit a number of adverse effects such as complement activation, prolonged PTT (partial thromboplastin time), thrombocytopenia, hepatotoxicity (elevation of liver enzymes), cardiotoxicity, splenomegaly and hyperplasia of reticuloendothelial cells.

As mentioned earlier, LNA provide unprecedented affinity, very high bio-stablity and the ability to modulate the exact molecular composition of the oligonucleotide. In one embodiment of the invention, LNA containing compounds enables the development of oligonucleotides which combine high potency with little- if any- phosphorothioate linkages and which are therefore likely to display better efficacy and safety than contemporary siRNA compounds.

MANUFACTURE

Oligo- and polynucleotides of the invention may be produced using the polymerisation techniques of nucleic acid chemistry well known to a person of ordinary skill in the art of organic chemistry. Generally, standard oligomerisation cycles of the phosphoramidite

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approach (S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, 49, 6123; S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, 48, 2223) is used, but *e.g.* H-phosphonate chemistry, phosphortriester chemistry can also be used.

For some monomers of the invention longer coupling time, and/or repeated couplings with fresh reagents, and/or use of more concentrated coupling reagents were used.
The phosphoramidites employed coupled with satisfactory >97% step-wise coupling yields. Thiolation of the phosphate is performed by exchanging the normal, *e.g.* iodine/pyridine/H_2O, oxidation used for synthesis of phosphordiester oligonucleotide with an oxidation using Beaucage's reagent (commercially available) other sulfurisation reagents are also comprised. The phosphorthioate LNA oligonucleotide were efficiently synthesised with stepwise coupling yields >= 97 %.

Purification of the individual siLNA strands was done using disposable reversed phase purification cartridges and/or reversed phase HPLC and/or precipitation from ethanol or butanol. Gel electrophoresis, reversed phase HPLC, MALDI-MS, and ESI-MS was used to verify the purity of the synthesized LNA oligonucleotides. Furthermore, solid support materials having immobilized thereto an optionally nucleobase protected and optionally 5'-OH protected LNA are especially interesting as material for the synthesis of the LNA oligonucleotides where an LNA monomer is included in at the 3' end. In this instance, the solid support material is preferable CPG, *e.g.* a readily (commercially) available CPG material or polystyrene onto which a 3'-functionalised, optionally nucleobase protected and optionally 5'-OH protected LNA is linked using the conditions stated by the supplier for that particular material.

One embodiment of the of the present invention is the novel method of synthesis of the siLNAs. It involves a method for the preparation of LNA oligonucleotides for use in siLNA, characterised by that the LNA monomers and RNA monomers are coupled using 1*H*-tetrazole or 5-ethylthio-1*H*-tetrazole. A further embodiment of this invention is that the method involves a coupling time, which is in the range of 400-1200s, preferably in 600-900s.

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The targets to be modified according to the present invention may be target is involved in a number of basic biological mechanisms including red blood cell proliferation, cellular proliferation, ion metabolism, glucose and energy metabolism, pH regulation and matrix metabolism. The invention described herein encompasses a method of preventing or treating cancer comprising a therapeutically effective amount of a target modulating siRNA compound to a human in need of such therapy.

PHARMACEUTICAL COMPOSITION

It should be understood that the invention also relates to a pharmaceutical composition, which comprises a least one siLNA construct of the invention as an active ingredient. It should be understood that the pharmaceutical composition according to the invention optionally comprises a pharmaceutical carrier, and that the pharmaceutical composition optionally comprises further siLNA compounds, chemotherapeutic compounds, anti-inflammatory compounds, antiviral compounds and/or immuno-modulating compounds.)

SALTS

The siLNA compound comprised in this invention can be employed in a variety of pharmaceutically acceptable salts. As used herein, the term refers to salts that retain the desired biological activity of the herein identified compounds and exhibit minimal undesired toxicological effects. Non-limiting examples of such salts can be formed with organic amino acid and base addition salts formed with metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, sodium, potassium, and the like, or with a cation formed from ammonia, *N,N*-dibenzylethylene-diamine, *D*-glucosamine, tetraethylammonium, or ethylenediamine; or (c) combinations of (a) and (b); e.g., a zinc tannate salt or the like.

PRODRUGS

In one embodiment of the invention the siLNA compound may be in the form of a pro-drug. Oligonucleotides are by virtue negatively charged ions. Due to the lipophilic nature of cell membranes the cellular uptake of oligonucleotides are reduced compared to

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neutral or lipophilic equivalents. This polarity "hindrance" can be avoided by using the pro-drug approach (see e.g. Crooke, R. M. (1998) in Crooke, S. T. *Antisense research and Application*. Springer-Verlag, Berlin, Germany, vol. 131, pp. 103-140). In this approach the oligonucleotides are prepared in a protected manner so that the oligonucleotide is neutral when it is administered. These protection groups are designed in such a way that so they can be removed then the oligonucleotide is taken up be the cells. Examples of such protection groups are S-acetylthioethyl (SATE) or S-pivaloylthioethyl (*t*-butyl-SATE). These protection groups are nuclease resistant and are selectively removed intracellulary.

CONJUGATES

In one embodiment of the invention the siLNA compound is linked to ligands/conjugates. It is way to increase the cellular uptake of siLNA. This conjugation can take place at the terminal positions 5'/3'-OH but the ligands may also take place at the sugars and/or the bases. In particular, the growth factor to which the siRNA oligonucleotide may be conjugated, may comprise transferrin or folate. Transferrin-polylysine-oligonucleotide complexes or folate-polylysine-oligonucleotide complexes may be prepared for uptake by cells expressing high levels of transferrin or folate receptor. Other examples of conjugates/lingands are

cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end-capping" with one or more nuclease-resistant linkage groups such as phosphoromonothioate, and the like.

The preparation of transferrin complexes as carriers of oligonucleotide uptake into cells is described by Wagner et al ., *Proc. Natl. Acad. Sci. USA* 87, 3410-3414 (1990). Low et al., U.S. Patent 5,108,921, describe cellular delivery of folate-macromolecule conjugates via folate receptor endocytosis, including delivery of the siRNA compound. Also see, Leamon et al., *Proc. Natl. Acad. Sci.* 88, 5572 (1991).

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FORMULATIONS

The invention also includes the formulation of one or more siLNA compound as disclosed herein. Pharmaceutically acceptable binding agents and adjuvants may comprise part of the formulated drug.

Capsules, tablets and pills etc. may contain for example the following compounds: microcrystalline cellulose, gum or gelatin as binders; starch or lactose as excipients; stearates as lubricants; various sweetening or flavouring agents. For capsules the dosage unit may contain a liquid carrier like fatty oils. Likewise coatings of sugar or enteric agents may be part of the dosage unit. The siLNA formulations may also be emulsions of the active pharmaceutical ingredients and a lipid forming a micellular emulsion.

A siLNA of the invention may be mixed with any material that do not impair the desired action, or with material that supplement the desired action. These could include other drugs including other nucleoside compounds.

For parenteral, subcutaneous, intradermal or topical administration the formulation may include a sterile diluent, buffers, regulators of tonicity and antibacterials. The active compound may be prepared with carriers that protect against degradation or immediate elimination from the body, including implants or microcapsules with controlled release properties. For intravenous administration the preferred carriers are physiological saline or phosphate buffered saline.

Preferably, a siLNA compound is included in a unit formulation such as in a pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount without causing serious side effects in the treated patient.

ADMINISTRATION

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be (a) oral (b) pulmonary, e.g., by inhalation or

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insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, (c) topical including epidermal, transdermal, ophthalmic and to mucous membranes including vaginal and rectal delivery; or (d) parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. In one embodiment the active oligonucleotide is administered IV, IP, orally, topically or as a bolus injection or administered directly in to the target organ.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Compositions and formulations for oral administration include but is not restricted to powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

DELIVERY

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self- emulsifying solids and self-emulsifying semisolids. Delivery of drug to tumour tissue may be enhanced by carrier-mediated delivery including, but not limited to, cationic liposomes, cyclodextrins, porphyrin derivatives, branched chain dendrimers,

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polyethylenimine polymers, nanoparticles and microspheres (Dass CR. J Pharm Pharmacol 2002; 54(1):3-27).

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels and suppositories. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

COMBINATION DRUG

siLNA compound are useful for a number of therapeutic applications as indicated above. In general, therapeutic methods of the invention include administration of a therapeutically effective amount of a siLNA to a mammal, particularly a human.

In a certain embodiment, the present invention provides pharmaceutical compositions containing (a) one or more siLNA compounds and (b) one or more other chemotherapeutic agents which function by a non-RNAi mechanism. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g. mithramycin and oligonucleotide), sequentially (e.g. mithramycin and oligonucleotide for a period of time followed by another agent and oligonucleotide), or in combination with one or more other such chemotherapeutic agents or in combination

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with radiotherapy. All chemotherapeutic agents known to a person skilled in the art are here incorporated as combination treatments with compound according to the invention.

Anti-inflammatory drugs, including but not limited to nonsteroidal anti- inflammatory drugs and corticosteroids, antiviral drugs, and immuno-modulating drugs may also be combined in compositions of the invention. Two or more combined compounds may be used together or sequentially.

Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, ibuprofen, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

In another embodiment, compositions of the invention may contain one or more siRNA compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional siRNA compounds. Two or more combined compounds may be used together or sequentially.

In another embodiment, siLNA compositions of the invention may contain one or more traditional antisense oligonucleotides targeted to the same target as the siLNA or targeted to a second nucleic acid target.

DOSAGE

Dosing is dependent on severity and responsiveness of the disease state to be treated, and the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient.

Optimum dosages may vary depending on the relative potency of individual oligonucleotides. Generally it can be estimated based on EC50s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 µg to 1 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 10 years or by continuous infusion for hours up to several months. The

repetition rates for dosing can be estimated based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state.

USES

The siLNA compounds of the present invention can be utilized for as research reagents for diagnostics, therapeutics and prophylaxis. In research, the siLNA may be used to specifically inhibit the synthesis of target genes in cells and experimental animals thereby facilitating functional analysis of the target or an appraisal of its usefulness as a target for therapeutic intervention. In diagnostics the siLNA oligonucleotides may be used to detect and quantitate target expression in cell and tissues by Northern blotting, in-situ hybridisation or similar techniques. For therapeutics, an animal or a human, suspected of having a disease or disorder, which can be treated by modulating the expression of target is treated by administering the siLNA compounds in accordance with this invention. Further provided are methods of treating an animal particular mouse and rat and treating a human, suspected of having or being prone to a disease or condition, associated with expression of target by administering a therapeutically or prophylactically effective amount of one or more of the siLNA compounds or compositions of the invention.

Example 1: Monomer synthesis

Preparation of the LNA monomers is described in great detail in the reference, Koshkin et al, *J. Org. Chem.*, **2001**, *66*, 8504-8512 and Pedersen et al., Synthesis, 2002, 6, 802-809 and references found therein, where the protection groups of Z and Z* are respectively oxy-N,N-diisopropyl-*O*-(2-cyanoethyl)phosphoramidite and dimethoxytrityloxy .

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Example 2: Oligonucleotide Synthesis (general)

All syntheses are carried out in 1 µmol scale on a MOSS Expedite instrument platform. The synthesis procedures are essentially carried out as described in the instrument manual.

Preparation of the LNA succinyl hemiester

5'-O-DMT-3'-hydroxy-LNA monomer (500 mg), succinic anhydride (1.2 eq.) and DMAP (1.2 eq.) were dissolved in DCM (35 mL). The reaction was stirred at room temperature overnight. After extractions with NaH_2PO_4 0.1 M pH 5.5 (2x) and brine (1x), the organic layer was further dried with anhydrous Na_2SO_4, filtered, and evaporated. The hemiester derivative was obtained in 95 % yield and was used without any further purification.

Preparation of the LNA-CPG (controlled pore glass)

The above prepared hemiester derivative (90 µmol) was dissolved in a minimum amount of DMF, DIEA and pyBOP (90 µmol) were added and mixed together for 1 min. This pre-activated mixture was combined with LCAA-CPG (500 Å, 80-120 mesh size, 300 mg) in a manual synthesizer and stirred. After 1.5 h at room temperature, the support was filtered off and washed with DMF, DCM and MeOH. After drying the loading was determined to be 57 µmol/g (see Tom Brown, Dorcas J.S.Brown. Modern machine-aided methods of oligodeoxyribonucleotide synthesis. In: F.Eckstein, editor. Oligonucleotides and Analogues A Practical Approach. Oxford: IRL Press, 1991: 13-14).

Phosphorothioate cycles

5'-O-DMT (A(bz), C(bz), G(ibu), and T) linked to CPG were deprotected using a solution of 3 % trichloroacetic acid (v/v) in dichloromethane. The CPG is washed with acetonitrile. Coupling of phosphoramidites (A(bz), G(ibu), 5-methyl-C(bz)) or T -β-cyanoethylphosphoramidite) is performed by using a solution of 0.08 M of the 5'-O-DMT-protected amidite in acetonitrile and activation is done by using DCI (4,5 – dicyanoimidazole) in acetonitrile (0.25 M). Coupling is carried out in 2 minutes. Thiolation is carried out by using Beaucage reagent (0.05 M in acetonitrile) and is

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allowed to react for 3 minutes. The support is thoroughly washed with acetonitrile and the subsequent capping is carried out by using the standard solution (CAP A) and (CAP B) to cap unreacted 5'- hydroxyl groups. The capping step is then repeated and acetonitrile washing concludes the cycle.

LNA unit cycles

5'-O-DMT A(bz), C(bz), G(ibu) or T linked to CPG is deprotected by using the same procedure as above. Coupling is performed by using 5'-O-DMT-A(bz), C(bz), G(ibu) or T-β-cyanoethylphosphoramidite (0.1 M in acetonitrile) and activation is done by DCI (0.25 M in acetonitrile). Coupling is prolonged to 7 minutes. Capping is done by using the standard solutions (CAP A) and (CAP B) for 30 sec. The phosphite triester is oxidized to the more stable phosphate triester by using a standart solution of I_2 and pyridine in THF for 30 sec. The support is washed with acetonitrile and the capping step is repeated. The cycle is concluded by thorough acetonitrile wash.

Cleavage and Deprotection

The oligonucleotide are cleaved from the support and the β-cyanoethyl protecting group removed by treating the support with 35 % NH_4OH 1 h at room temperature. The support is filtered off and the base protecting groups are removed by raising the temperature to 65 °C for 4 hours. The ammonia is then removed by evaporation.

Purification

The oligos are either purified by (reversed-phase) RP-HPLC or (anion exchange) AIE.

RP-HPLC:

Column: VYDAC™ cat. No. 218TP1010 (vydac)

Flow rate: 3 mL/min

Buffer: A 0.1 M ammonium acetate pH 7.6

B acetonitrile

Gradient:

Time	0	10	18	22	23	28
B %	0	5	30	100	100	0

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IE:

Column: Resource™ 15Q (*amersham pharmacia biotech*)

Flow rate: 1.2 mL/min

Buffer: A 0.1 M NaOH

B 0.1 M NaOH, 2.0 M NaCl

Gradient:

Time	0	1	27	28	32	33
B %	0	25	55	100	100	0

Abbreviations

DMT:	Dimethoxytrityl
DCI:	4,5-Dicyanoimidazole
DMAP:	4-Dimethylaminopyridine
DCM:	Dichloromethane
DMF:	Dimethylformamide
THF:	Tetrahydrofuran
DIEA:	*N,N*-diisopropylethylamine
PyBOP:	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
Bz:	Benzoyl
Ibu:	Isobutyryl
Beaucage:	3H-1,2-Benzodithiole-3-one-1,1-dioxide
A(bz), C(bz), G(ibu), T:	LNA-monomers (LNA-locked nucleic acid)

Tm measurement

Melting curves were recorded with a Perkin Elmer UV/Vis spectrophotometer lambda 40 attached to a PTP-6 Peltier System. Oligonucleotides were dissolved in salt buffer (10 mM phosphate buffer, 100 mM NaCl, 0,1 mM EDTA, pH 7.0) using the two complementary strands at 1.5 µM and 1 cm path-length cells. Samples were denatured at 95°C for 3 min and slowly cooled to 20°C prior to measurements. Melting curves were recorded at 260 nm using a heating rate of 1°C/min, a slit of 2 nm and a response of 0.2 s. Tm values were obtained from the maxima of the first derivatives of the melting curves.

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Example 3: Synthesis of LNA/RNA oligonucleotides

Synthesis

LNA/RNA oligonucleotides were synthesized DMT-off on a 1.0 µmol scale using an automated nucleic acid synthesizer, MOSS Expedite 8909, using standard reagents. Either 1*H*-tetrazole or 5-ethylthio-1*H*-tetrazole was used as activators. LNA A^{Bz}, G^{tBu} and T phosphoramidite concentration was 0.1 M in anhydrous acetonitrile. The $^{Me}C^{Bz}$ was dissolved in 15 % THF in acetonitrile. The coupling time for all monomer couplings was 600 s. The RNA phosphoramidites (Glen Research, Sterling, Virginia) were *N*-acetyl and 2'-*O*-triisopropylsilyloxymethyl (TOM) protected. The monomer concentration was 0.1 M (anhydrous acetonitrile) and the coupling time was 900 s. The oxidation time was set to 50 s. The solid support was DMT-LNA-CPG (1000 Å, 30-40 µmol/g).

Work-up and Purification

Cleavage from the resin and nucleobase/phosphate deprotection was carried out in a sterile tube by treatment with 1.5 mL of a methylamine solution (1:1, 33 % methylamine in ethanol: 40 % methylamine in water) at 35°C for 6 h or left overnight. The tube was centrifuged and the methylamine solution was transferred to another sterile tube. The methylamine solution was evaporated in a vacuum centrifuge. To remove the 2'-*O*-protection groups the residue was dissolved in 1.0 mL 1.0 M TBAF in THF and heated to 55°C for 15 min and left at 35°C overnight. The THF was evaporated in a vacuum centrifuge leaving a light yellow gum, which was neutralized with approx. 600 µL (total sample volume: 1.0 mL) of RNase free 1.0 M Tris-buffer (pH 7). The mixture was homogenized by shaking and heating to 65°C for 3 min.

Desalting of the oligonucleotides was performed on NAP-10 columns (Amersham Biosciences, see below). The filtrate from step 4 was collected and analysed by MALDI-TOF and gel electroforesis (16 % sequencing acrylamide gel (1 mm), 0.9 % TBE [Tris: 89 mM, Boric acid: 89 mM, EDTA: 2 mM, pH 8.3] buffer, ran for 2 h at 20 W as the limiting parameter. The gel was stained in CyberGold (Molecular Probes, 1:10000 in

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0.9xTBE) for 30 min followed by scanning in a Bio-Rad FX Imager). The concentration of the oligonucleotide was measured by UV-spectrometry at 260 nm.

Scheme A, Desalting on NAP-10 columns:

Step	Reagent	Operation	Volume	Remarks
1	-	Empty storage buffer	-	Discard
2	H_2O (RNase free)	Wash	2 x full volume	Discard
3	Oligo in buffer (RNase free)	Load	1.0mL	Discard
4	H_2O (RNase free)	Elution	1.5mL	Collect – Contains oligo
5	H_2O (RNase free)	"Elution"	0.5mL	Collect – Contains salt + small amount of oligo

The most important issues in the synthesis of LNA/RNA are:

- Extended coupling times, compared to the standard procedure, are necessary to achieve good coupling efficiency.
- Oxidation time have to be extended, compared to the standard procedure, to minimize the formation of deletion fragments.
- Coupling of 2'-*O*-TOM protected phosphoramidites are superior to the 2'-*O*-TBDMS counterparts according to the trityl data.

With these improvements the crude oligonucleotides are of such quality that further purification can be avoided. MS analysis should be carried out after the TOM-groups are removed.

Example 3 SiLNA improved stability compared to siRNA

SiLNA improved stability compared to siRNA (Figure 2). Both lightly and more heaily modified siLNA clearly improved stability. Stability was evaluated in 10% foetal bovine serum diluted in physiological saline solution. The siRNA and siLNA were incubated in the serum at 37°C. Samples were withdrawn at different time points. The samples were

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analyzed on 15 % polyacrylamide TBE gels and stained with SYBR-gold (Molecular probes).

Example 4: Test of design of the siLNA compound in mammalian reporter system

Efficacy of different siLNA designs and combinations are first assessed in a luciferase reported system in mammalian cell culture. Oligonucleotides used are listed in table 1. Sense and corresponding antisense oligonucleotides are hybridized to generate double strands, ie. siRNA or siLNA.

Cells used were the human embryonal kidney (HEK) 293 cell lines. HEK 293 cells were maintained in DMEM supplemented with 10 % foetal bovine serum, penicillin, streptomycine and glutamine (Invitrogen, Paisely, UK).

The plasmids used were pGL3-Control coding for firefly luciferace under the control of the SV40 promoter and enhancer and pRL-TK coding for *Renilla* luciferase under the control of HSV-TK promoter (Promega, Madison, WI, USA).

Transfection

Cells were seeded in 500 µl medium in 24-well plates one day before transfection to adhere and reach a confluency of 70 to 90 percent at the time of transfection. Cells were seeded in medium without antibiotics and changed to 500 µl Opti-MEM I just before adding the transfection mix to the cells.

Standard co-transfection mix was prepaired for triplicate wells by separately adding 510 ng pGL3-Control, 51 ng pRL-TK and 340 ng siRNA to 150 µl Opti-MEM I (Invitrogen) and 3 µl LipofectAMINE 2000 (Invitrogen) to another 150 µl Opti-MEM I. The two solutions were mixed and incubated at room temperature for 20-30 minutes before adding to the cells. 100 µl of the transfection mix were added to each of three wells. Final volume of medium plus transfection mix was 600 µl. The siLNA or siRNA concentration corresponds to about 13 nM. Cells are incubated with the transfection mix for 4 hours and the medium is then changed for new DMEM fully supplemented.

Dual-Luciferase Reporter Assay (Promega)

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Cells were harvested in passive lysis buffer and assayed according to protocol (Promega) using a NovoSTAR 96-well format luminometer with substrate dispenser (BMG Labtechnologies, Offenburg, Germany). 10 µl sample were applied in each well of a 96 well plate and 50 µl Luciferace Assay Reagent II (substrate for firefly luciferase) were added to a well by the luminometer and measured. Then 50 µl Stop and Glow (stop solution for firefly luciferase and substrate for *Renilla* luciferase) were added and measured. Averages of the luciferase activities measured for 10 seconds were used to calculate ratios between firefly and *Renilla* luciferase or the opposite.

Example 5: *In vitro* model: Cell culture assessing efficacy on endogenous target

Cells used were the the rat adrenal pheochromocytoma, PC12 cell lines. PC12 were maintained in DMEM supplemented with 10 % horse serum, 5 % foetal bovine serum, penicillin, streptomycine and glutamine.

SiLNA or siRNA transfection protocol for endogenous genes (like NPY in PC12 cells) follow the same procedure as above but without adding plasmids only siRNA. Final si-LNA or siRNA concentrations ranged from 1 to 100 nM. Cells were usually harvested 24 to 48 hours post transfection and mRNA extracted. mRNA levels were measured with Northern blot or Real-Time PCR. See the siLNA down regulation of the NPY target in PC12 in Figure 3.

Example 6: *In vitro* model: Analysis of Oligonucleotide Inhibition of target *Expression by Real-time PCR*

SiLNA or siRNA gene silencing of Target can be assayed in a variety of ways known in the art. For example, Target mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymetargete chain reaction (PCR), or real-time PCR. Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or mRNA.

Methods of RNA isolation and RNA analysis such as Northern blot analysis is routine in the art and is taught in, for example, Current Protocols in Molecular Biology, John Wiley and Sons.

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Cells were harvested and mRNA was extracted. Standard real-time PCR protocols were used to amplify target genes from mRNA with gene specific primers along with a primer pair towards a housekeeping gene as internal control (such as Cyclophilin). Down-regulation was expressed as a ratio of amount target mRNA to amount control mRNA.

Real-time quantitative (PCR) can be conveniently accomplished using the commercially available iQ Multi-Color Real Time PCR Detection System, available from BioRAD.

Example 7: *in vitro* analysis: Northern Blot Analysis of Target mRNA Levels

Northern blot analysis was carried out by procedures well known in the art essentially as described in Current Protocols in Molecular Biology, John Wiley & Sons
Cells were harvested and mRNA was extracted. Standard northern protocol was followed. mRNA was separated on a denaturing agarose gel, transfered to a membrane and probed with target gene specific probes (such as NPY probes) and as internal control a probe towards a housekeeping gene was used (such as Actin) served as internal control. Down-regulation was expressed as a ratio of amount target mRNA to amount control mRNA.

Example 8: *In vitro* analysis: Western blot analysis of Target protein levels

Protein levels of Target can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA, RIA (Radio Immuno Assay) or fluorescence-activated cell sorting (FACS). Antibodies directed to Target can be identified and obtained from a variety of sources, such as Upstate Biotechnologies (Lake Placid, USA), Novus Biologicals (Littleton, Colorado), Santa Cruz Biotechnology (Santa Cruz, California) or can be prepared via conventional antibody generation methods.

Example 9; *In vitro* analysis: SiRNA Inhibition of Reporter Target Expression by siLNA oligonucleotides

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LNA monomers could be used to modify both ends of the sense strand in siLNA with maintained effect compared to siRNA, >90 % inhibition of Firefly Luciferase expression compared to untreated samples. The antisense strand could also be modified in the 3'-end without loss of efficiency while a modification in the 5'-end of the antisense strand reduced the effect to 25-70 % inhibition. Further modifications, exchanging all uracil to LNA thymines in the sense strand reduced the effect to 80 % inhibition. A similar modification of the antisense strand abolished the effect (Figure 4 and 5). Phosphorylation of the 5'-end of the siLNA antisense strand did not improve the reduction (20-30% reduction) (data not shown). Similar experiments targeting Renilla Luciferase showed that both ends of the sense strand could be modified with LNA monomers while the antisense strand tolerates 3'-end LNA monomer modification (95 % inhibition in all cases) but showed less inhibition with both a 3' and 5'-end LNA modification. Still there was an up to 75 % inhibition (Figure 5). Single stranded antisense RNA has showed up to 50 % reduction of firefly luciferase expression but corresponding LNA monomer contained single strand has only given up to 30 % reduction. In the case where all uracil have been exchanged to LNA thymidines showed the highest reduction (30%) (data not shown). Stability of single strands has only been accessible with the all uracil to thymidine ON, where the stability was similar to naked DNA. Less modified single strands and unmodified double strand were degraded already in the time point zero sample (Figure 6).

Example 10 *In vitro* analysis: SiRNA Inhibition of endogenious target by siLNA oligonucleotides

Inhibition of cytotoxicity: Cells were transfected with 85 nM of the respective siRNA or siLNA (SARS 1-4, see Figure 7) or with control siRNA targeting the firefly luciferase gene (Luc) or the rat neuropeptide Y (NPY) gene. Mock-transfected cells treated with Lipofectamine 2000 only and used as positive control. Uninfected cells were included as negative control. Transfected cells were infected with either 60000, 6000 or 600 $TCID_{50}$ of SARS-CoV. After 50 hours of infection, the CPE and the cytotoxicity were measured. There was a marked difference in CPE between the cells treated with the most effective siRNA, SARS 1, as compared to mock-transfected cells (Figure 8).

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The cytotoxicity was determined as percent LDH release from treated cells as compared to mock-transfected control cells. The percent inhibition of cytotoxicity was calculated as 100 – percent cytotoxicity in the siRNA treated sample. The four Pol-specific siRNA and siLNA had various effects on cytotoxicity (Figure 9). The most effective siRNA and siLNA were the ones targeting site SARS 1, which reduced cytotoxicity with up to 65 % at 600 $TCID_{50}$. The SARS 3 site was medium efficient using siRNA at all three viral doses. However SARS 3 became an equally efficient site as SARS 1 by using siLNA, also att all three viral doses. The sites SARS 2 and SARS 4 did no show any effect by siRNA or siLNA at any viral dose. The data represent mean and standard deviation of three independent experiments in quadruplicate.

Virus and cells: Vero cells were used for all cellular experiments. Cells were cultivated in phenol red-free Eagle's MEM containing 5 % FCS and 1 % PEST at 37°C and 5 % CO_2. The Frankfurt 1 isolate (GenBank accession number AY291315, kindly provided by Dr. H. W. Doerr) was grown to high titers on Vero cells. Supernatants from two T225 cell culture flasks were pooled and frozen at -80°C in 1 ml vials and constituted the viral stock. The stock virus was identified as SARS-CoV by diagnostic reverse transcriptase PCR using the BNIoutS2 and BNIoutAs [11] primers and the Cor-p-F2 and Cor-p-R1 primers [2]. The virus stock was used in ten-fold dilutions or at a fixed dilution to infect Vero cells in 96 well cell culture plates. The virus stock was diluted 600,000 times (determined by the Reed-Muench method) to reach $TCID_{50}$ in 96 well cell culture plates. The siLNA oligonucleotides were produced according to above. Sequence are shown in figure 7.

Tranfections: Lipofectamine2000 (Invitrogen) was used to transefect the cells with siRNA and siLNA. Transfection efficiency was high and most cell were transfected. The transfection medium was changed to phenol red-free Eagle's MEM after four hours, and cells were grown over night to form a confluent monolayer.

Cytopathogenicity and cytotoxicity: The cytopathogenic effect (CPE) on infected cells was detected as cell rounding and detachment from the cell culture plate. The CPE was scored in a light microscope. The cytotoxicity was measured using a cytotoxicity detection kit (LDH) (Roche, Germany). Mock-transfected cells treated with

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lipofectamine2000 only were set as 100 % cytotoxicity caused by the virus infection at each viral dilution. Uninfected cells were used to determine the background cytotoxicity. The percent cytotoxicity was determined as [(Abs490 sample – background/Abs490 mock-transfected controls – background)×100]. The inhibition of cytotoxicity was calculated as [(1-Abs490 sample – background/Abs490 mock-transfected controls – background)×100].

Example 10: *In vivo* efficacy of LNA modified siRNA using the GFP trangenic mouse model

Initial trials will use siLNA together with transgenic mouse embryonal stem cells. In these cells yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) are expressed and the siLNA are targeting these genes. Detection of down-regulation is primarily done by fluorescent microscopy. CFP and YFP are very similar to GFP and the same sequence as green fluorescent protein (GFP).

Initial down regulation of YFP/CFP by si-LNA/RNA is verified in the mouse embryonal stem cells.

Verified efficient siRNA sequences are used in the GFP transgenic mouse model to show *in vivo* efficacy of siLNA. We access the GFP transgenic mouse model, which is ubiquitously expressing GFP.

SiLNA will be injected stereotactic in the brain in various nuclei and GFP down regulation is examined. Whole animal delivery, intravenously show tissue specific down regulation of GFP, in organs such as liver, kidney, for example. Local administration, for example intramuscularly, directly in the liver, etc., will show local down regulation.

Detection of GFP in vivo.

The whole animal is frozen and cryosectioned alternatively perfused with formalin and sectioned. The distribution of GFP down regulation is detected by fluorescent microscopy (active protein), in situ hybridization (mRNA level) and antibody staining (protein level).

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In vivo delivery of siLNA might require carrier agents such as chitosan, polyethyleneimine (PEI), various liposomal formulations, etc.

Example 11: Biodistribution of siLNA compounds in mice

Female NMRI athymic nude mice of 6 weeks old were purchased from M&B, Denmark and allowed to acclimatize for at least one week before entering experiments. Human cancer cells typically 10^6 cells suspended in 300 µl matrigel (BD Bioscience) were subcutaneously injected into the flanks of 7-8 week old NMRI athymic female nude mice. When tumour growth was evident, tritium labelled oligonucleotides were administrated at 5 mg/kg/day for 14 days using ALZET osmotic pumps implanted subcutaneously. The oligonucleotides were tritium labeled as described by Graham MJ et al. (J Pharmacol Exp Ther 1998; 286(1): 447-458). Oligonucleotides were quantitated by scintillation counting of tissue extracts from all major organs (liver, kidney, spleen, heart, stomach, lungs, small intestine, large intestine, lymph nodes, skin, muscle, fat, bone, bone marrow) and subcutaneous transplanted human tumour tissue.

Claims

What is claimed is:

1. A double-stranded compound, containing RNA, DNA, nucleic acid analogues or a combination thereof, wherein each strand has a length from 12-35 nucleotides, wherein said compound is capable of target-specific nucleic acid modifications, and wherein the compound consists of at least one LNA monomer.

2. A compound according to claim 1, where in the compound is a siLNA.

3. The siLNA compound of any one of claims 1-2, which contains at least one LNA monomer of the following formula:

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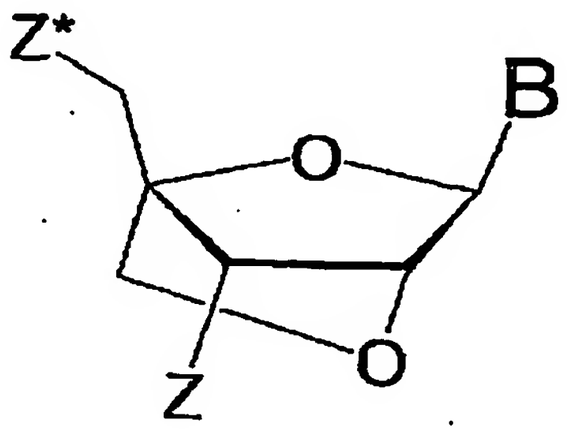

Z and Z* are independently selected among an internucleoside linkage, a terminal group or a protecting group and

B constitutes a natural or non-natural nucleobase and selected among adenine, cytosine, 5-methylcytosine, isocytosine, pseudoisocytosine, guanine, thymine, uracil, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, 2,6-diaminopurine, 2-chloro-6-aminopurine.

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4. The siLNA compound of any one of claims 1-3, wherein the internucleoside may be selected from the group consisting of -O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -S-P(O)$_2$-O-, -S-P(O,S)-O-, -S-P(S)$_2$-O-, -O-P(O)$_2$-S-, -O-P(O,S)-S-, -S-P(O)$_2$-S-, -O-PO(R^H)-O-, O-PO(OCH$_3$)-O-, -O-PO(NR^H)-O-, -O-PO(OCH$_2$CH$_2$S-R)-O-, -O-PO(BH$_3$)-O-, -O-PO(NHR^H)-O-, -O-P(O)$_2$-NR^H-, -NR^H-P(O)$_2$-O-, -NR^H-CO-O-, -NR^H-CO-NR^H-, -O-CO-O-, -O-CO-NR^H-, -NR^H-CO-CH$_2$-, -O-CH$_2$-CO-NR^H-, -O-CH$_2$-CH$_2$-NR^H-, -CO-NR^H-CH$_2$-, -CH$_2$-NR^H-CO-, -O-CH$_2$-CH$_2$-S-, -S-CH$_2$-CH$_2$-O-, -S-CH$_2$-CH$_2$-S-, -CH$_2$-SO$_2$-CH$_2$-, -CH$_2$-CO-NR^H-, -O-CH$_2$-CH$_2$-NR^H-CO -, –CH$_2$-NCH$_3$-O-CH$_2$-, where R^H is selected form hydrogen and C$_{1-4}$-alkyl,

5. The siLNA compound of any one of claims 1-4, wherein at least one LNA monomer is incorporated in the sense strand.

6. The siLNA compound of any one of claims 1-6, wherein at least one LNA monomer is incorporated in the antisense strand.

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7. The siLNA compound of any one of claims 1-7, wherein at least one LNA monomer is incorporated in the sense strand and at least one LNA monomer is incorporate in the antisense strand.

8. The siLNA compound of any one of claims 1-7, wherein the LNA monomer(s) are incorporated in at least one of the 3'-overhangs.

9. The siLNA compound of any one of claims 1-8, wherein the at least on LNA monomer is incorporated in any positions except the 5'-end of the antisense strand.

10. The siLNA compound of any one of claims 1-9, wherein at least one strand has a 3'-overhang from 0-7 nucleotides, particularly 1-3 nucleotides.

11. The siLNA compound of any of claims 1-10, wherein at least one strand has a 5'-overhang from 0-4 nucleotides, particularly 1-3 nucleotides.

12. The siLNA compound of any one of claims 1-11, capable of target-specific RNA interference.

13. The siLNA compound of any one of claims 1-12, wherein each strand has a length from 17-25, particularly from 20-22 nucleotides.

14. The siLNA compound of any one of claims 1-13, where in 5-90% of the nucleotides constitute of LNA monomers.

15. The siLNA compound of any one of claims 1-14, wherein the compound is modified with at least one RNA.

16. The siLNA compound of any one of claims 1-15, wherein the compound is modified with at least one DNA.

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17. The siLNA compound of any one of claims 1-16, wherein the compound is modified with at least one nucleic acid analogue.

18. The siLNA compound of any one of claims 17, wherein the nucleic acid analogue is selected from phosphorthioate, 2'-O-Methyl, 2'-MOE, 2'-Flouro, 2'-AP, 2'-F-ANA, 2''-(3-hydroxy)propyl, boranophosphates, 3'-phosphoramidate, HNA, CeNA, Morpholino, PNA.

19. The siLNA compound of any one of claims 1-18, wherein the antisense sequence has least 70 percent, particularly 90-100% sequence identity to the target molecule.

20. The siLNA compound of any one of claims 1-19, wherein the target molecule is a RNA target.

21. The siLNA compound of claims 20, wherein the target molecule is selected from the group of genomic RNA, genomic viral RNA, mRNA or a pre-mRNA.

22. The siLNA compound of any one of claims 1-21, wherein target gene is a gene associated with a pathological condition, a viral gene, a cancer related gene, a tumor-associated gene or an autoimmune disease-associated gene.

23. A method of preparing a siLNA of any one of claims 1-22, comprising the steps: (a) synthesizing two strands each having a length from 19-25 nucleotides, wherein said strands are capable of forming a double-stranded molecule, (b) combining the synthesized strands under conditions, wherein a double- stranded molecule is formed, which is capable of target specific nucleic acid modifications.

24. A method of mediating target-specific nucleic acid modifications in a cell or an organism comprising the steps: (a) contacting said cell or organism with the siLNA compound of any one of claims 1- 22 under conditions wherein target-specific nucleic acid modifications can occur, and (b) mediating a target-specific nucleic acid

modification effected by the siLNA compound towards a target nucleic acid having a sequence portion substantially corresponding to the siLNA compound.

25. The method of claim 23 or 24, wherein the nucleic acid modification is RNA interference.

26. The method of any of the claims 23-25, wherein said contacting comprises introducing said siLNA molecule into a target cell in which the target-specific nucleic acid modification can occur.

27. The method of any of the claims 23-26, wherein the introducing comprises a carrier mediated delivery or injection.

28. Use of the method of any one of claims 23-27, for determining the function or modulating of a gene in a cell or an organism.

29. Use of a method of any of the above claims 23-27, wherein the target to be modified is a gene is associated with a pathological condition, a viral gene, a cancer related gene, a tumor-associated gene or an autoimmune disease-associated gene.

30. Pharmaceutical composition containing as an active agent at least one siLNA compound of any one of claims 1-22 and a pharmaceutical carrier.

31. The composition of claim 30 for therapeutic applications.

32. Pharmaceutical composition containing as an active agent at least one siLNA compound of any one of claims 1-22 in combination at least one more active agent.

33. A method for the preparation of LNA oligonucleotide(s) for use in a siLNA compound according to claim 1-20, wherein LNA monomers and RNA monomers are coupled using 1*H*-tetrazole or 5-ethylthio-1*H*-tetrazole.

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34. A method according to claim 33, wherein the coupling time is from 400-1200s, preferably in 600-900.

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ABSTRACT

The present invention provides compositions and methods for double-stranded RNA (dsRNA) that induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi). The dsRNA according to the present invention e.g. has enhanced properties through the utilisation of LNA (Locked Nucleic Acid).

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Table 1

Following LNA/RNA oligonucleotides where synthesized:

Oligo Cureon No	Sequence (5'→3')	Conc. (µM)	Ap-prox. purity
2184	rC rU rU rA rC rG rC rU rG rA rG rU rA rC rU rU rC rG rA T T	440	~80%
2185	^{Me}C rU rU rA rC rG rC rU rG rA rG rU rA rC rU rU rC rG rA T T	320	~70%
2186	rU rC rG rA rA rG rU rA rC rU rC rA rG rC rG rU rA rA rG T T	380	~65%
2187	T rC rG rA rA rG rU rA rC rU rC rA rG rC rG rU rA rA rG T T	340	~60%
2187-phos	Phos-T rC rG rA rA rG rU rA rC rU rC rA rG rC rG rU rA rA rG T T	350	~80%
2188	^{Me}C T T rC rG rC T rA rG T rA rC T T rC rG rA T T	410	~50%
2189	T rC rG rA rA rG T rA rC T rC rA rG rC rG T rA rA rG T T	390	~55%
2189-phos	Phos-T rC rG rA rA rG T rA rC T rC rA rG rC rG T rA rA rG T T	330	~80%
2699-1	rU rU rU rU rU rC rU rC rC rU rU rC rU rU rC rA rG rA rU T T	400	~80%
2700-1	rA rU rC rU rG rA rA rG rA rA rG rG rA rG rA rA rA rA rA T T	400	~80%
2701-1	T rU rU rU rU rC rU rC rC rU rU rC rU rU rC rA rG rA rU T T	360	~80%
2702-1	A rU rC rU rG rA rA rG rA rA rG rG rA rG rA rA rA rA rA T T	430	~80%
2703-1	^{Me}C T T rA rC rG rC T rG rA rG T rA rC T T rC rG rA T T	500	~80%

Capital letters: LNA; Small letters (with an "r" prefix): RNA; Phos: 5'-phosphate.

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Table 2

Cur no.	Sequence (5'→3')
2780	$^{m}C^{o}$ T^{o} T^{o} A^{o} $^{m}C^{o}$ G^{o} $^{m}C^{o}$ T^{o} G^{o} A^{o} G^{o} T^{o} A^{o} $^{m}C^{o}$ T^{o} T^{o} $^{m}C^{o}$ G^{o} A^{o} T^{o} T^{o}
2781	dc T^{o} dt A^{o} dc G^{o} dc T^{o} dg A^{o} dg T^{o} da $^{m}C^{o}$ dt T^{o} dc G^{o} da T^{o} T^{o}
2782	dc T^{o} dt da $^{m}C^{o}$ dg dc T^{o} dg da G^{o} dt da $^{m}C^{o}$ dt dt $^{m}C^{o}$ dg da T^{o} T^{o}
2783	$^{m}C^{o}$ ru ru A^{o} rc G^{o} rc ru G^{o} ra G^{o} ru ra $^{m}C^{o}$ ru ru $^{m}C^{o}$ rg ra T^{o} T^{o}
2784	$^{m}C^{o}$ du du A^{o} dc G^{o} dc du G^{o} da G^{o} du da $^{m}C^{o}$ du du $^{m}C^{o}$ dg da T^{o} T^{o}
2785	ru $^{m}C^{o}$ G^{o} A^{o} A^{o} G^{o} T^{o} A^{o} $^{m}C^{o}$ T^{o} $^{m}C^{o}$ A^{o} G^{o} $^{m}C^{o}$ G^{o} T^{o} A^{o} A^{o} G^{o} T^{o} T^{o}
2786	ru $^{m}C^{o}$ dg A^{o} da G^{o} dt A^{o} dc T^{o} dc A^{o} dg $^{m}C^{o}$ dg T^{o} da A^{o} dg T^{o} T^{o}
2787	ru $^{m}C^{o}$ dg da A^{o} dg dt A^{o} dc dt $^{m}C^{o}$ da dg $^{m}C^{o}$ dg dt A^{o} da dg T^{o} T^{o}
2788	ru rc G^{o} ra ra G^{o} ru ra $^{m}C^{o}$ ru rc A^{o} rg rc G^{o} ru A^{o} ra rg T^{o} T^{o}
2789	ru $^{m}C^{o}$ rg ra ra rg ru ra rc ru rc ra rg rc rg ru ra ra rg T^{o} T^{o}
2790	ru rc G^{o} ra ra rg ru ra rc ru rc ra rg rc rg ru ra ra rg T^{o} T^{o}
2792	ru rc rg ra A^{o} rg ru ra rc ru rc ra rg rc rg ru ra ra rg T^{o} T^{o}
2793	ru rc rg ra ra G^{o} ru ra rc ru rc ra rg rc rg ru ra ra rg T^{o} T^{o}
2794	ru rc rg ra ra rg ru A^{o} rc ru rc ra rg rc rg ru ra ra rg T^{o} T^{o}
2795	T^{o} rg A^{o} rg A^{o} rg ra ra A^{o} rg rc A^{o} rc A^{o} rg ra A^{o} ra ra T^{o} T^{o}
2796	T^{o} rg ra rg ra rg ra ra ra rg rc ra rc ra rg ra ra ra ra T^{o} T^{o}
2797	T^{o} ru ru ru rc ru rg ru rg rc ru ru ru rc ru rc ru rc ra T^{o} T^{o}

Capital letters: LNA.

Small letters (with an "r" prefix): RNA.Small letters (with an "d" prefix): DNA

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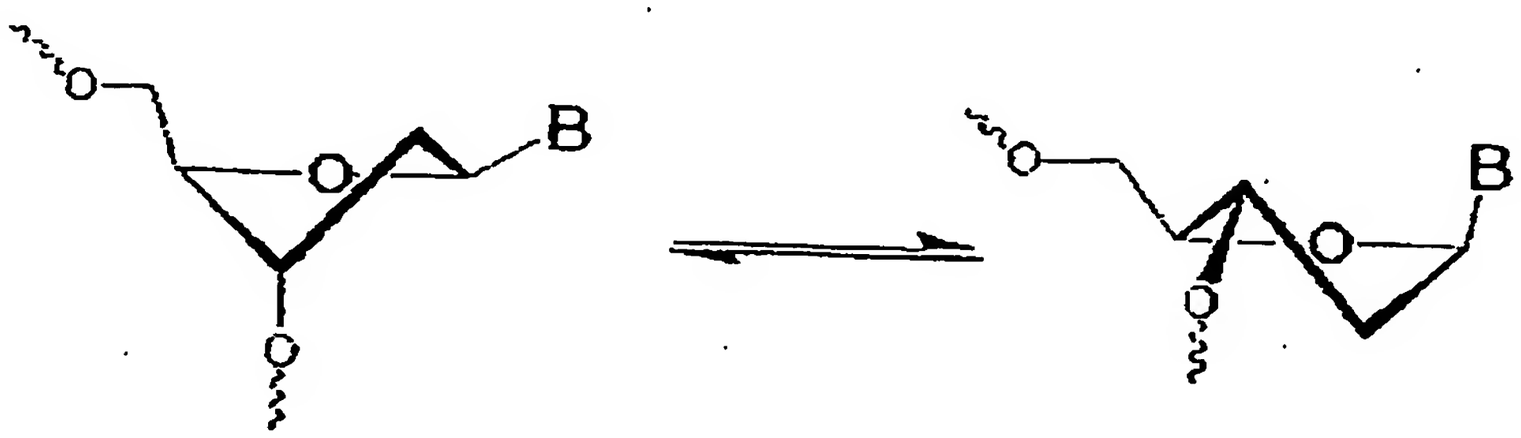
Figure 1/10



C2'-endo (S-type) C3'-endo (N-type)

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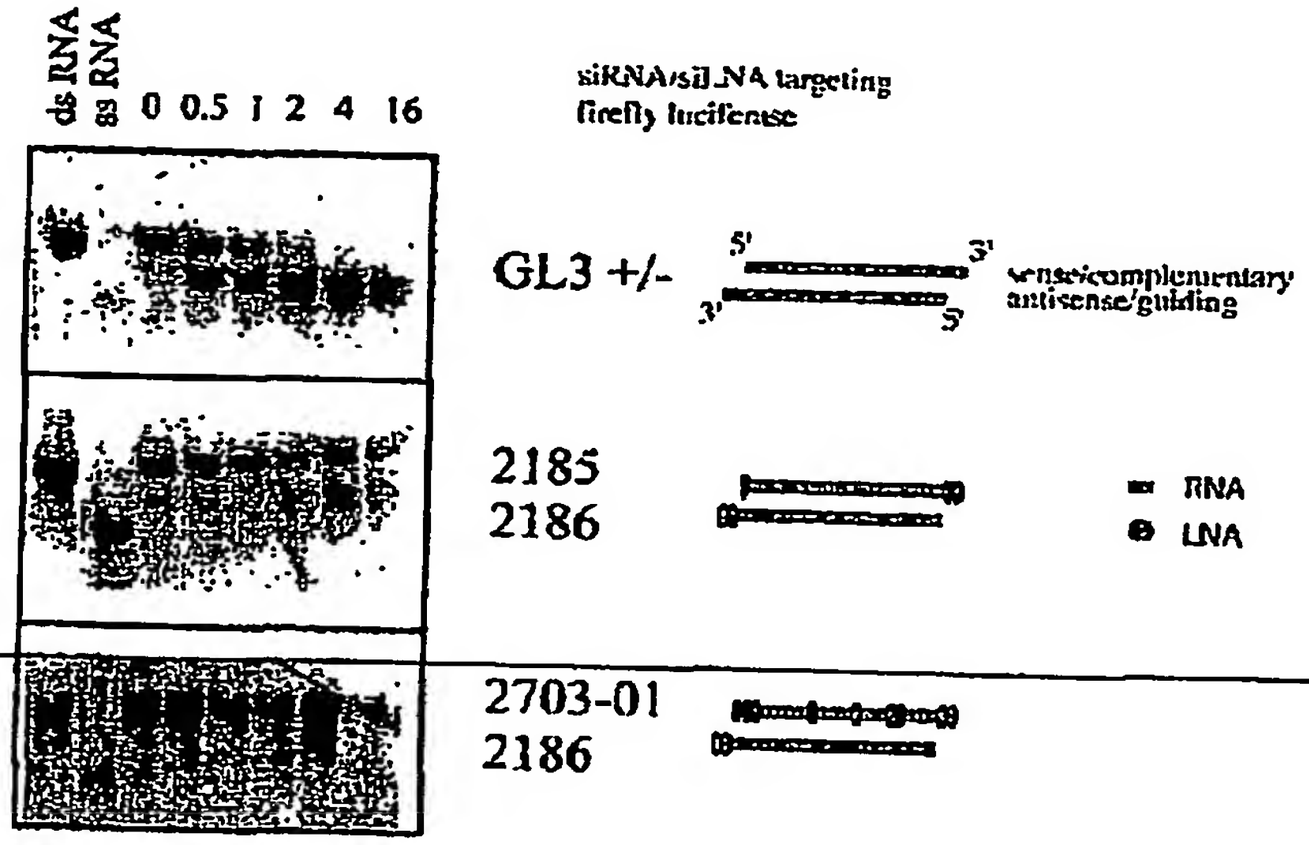
Figure 2/10

Improved stability of siLNA over siRNA.



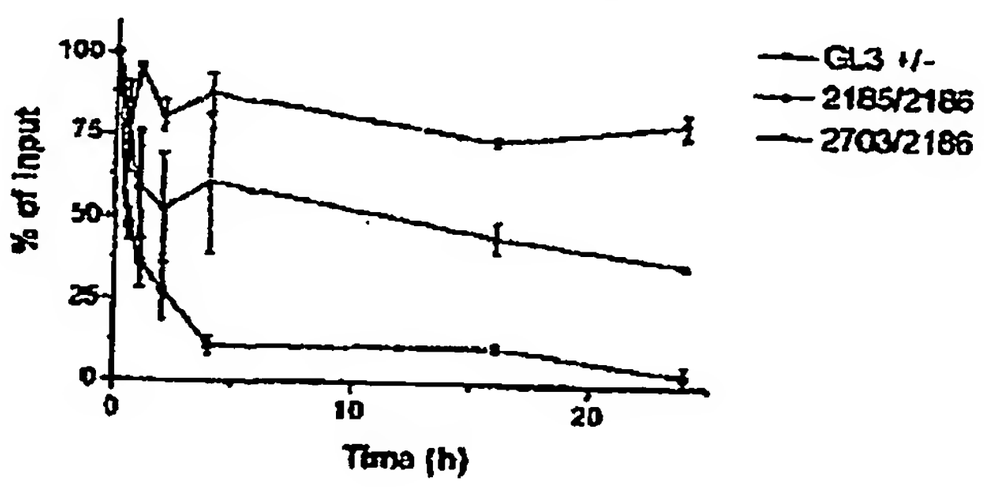


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Figure 3/10

Preliminary data (mRNA levels, Q-PCR), down regulating the endogenous gene NPY in PC12 cells (21/5-03).

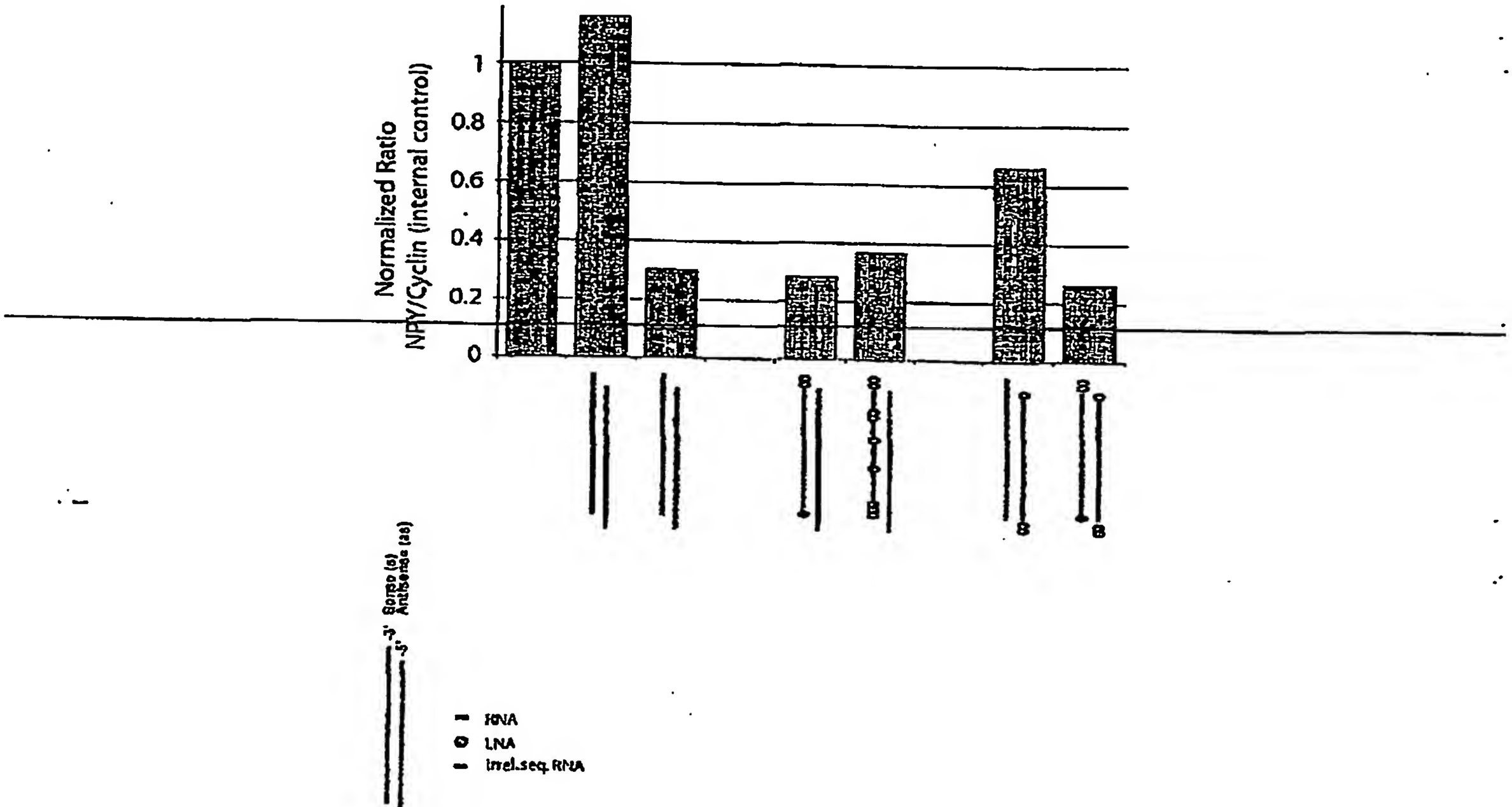

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Figure 4/10

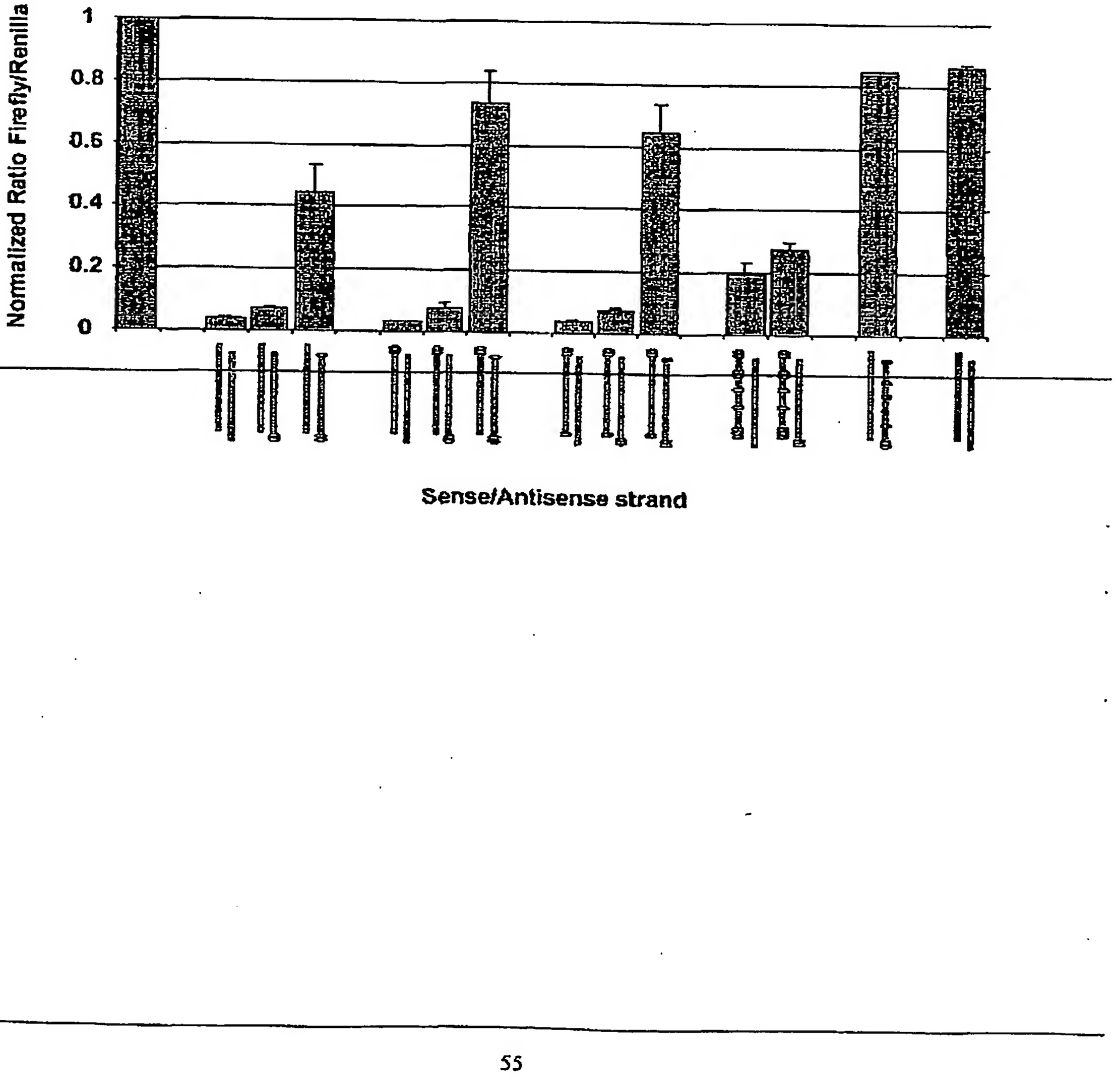

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Figure 5/10

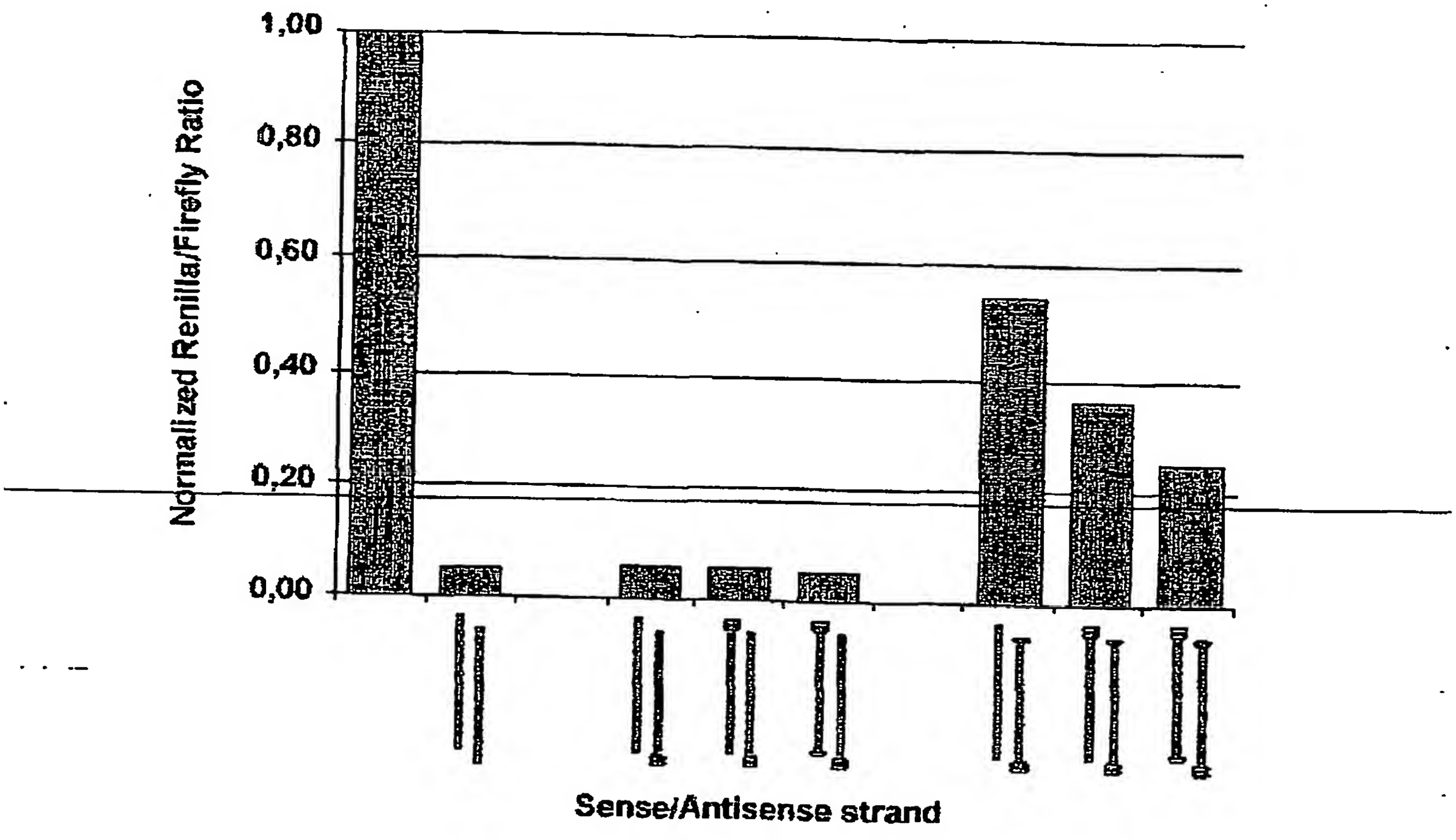

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Figure 6/10

Preliminary stability of ssLNA/RNA ONs

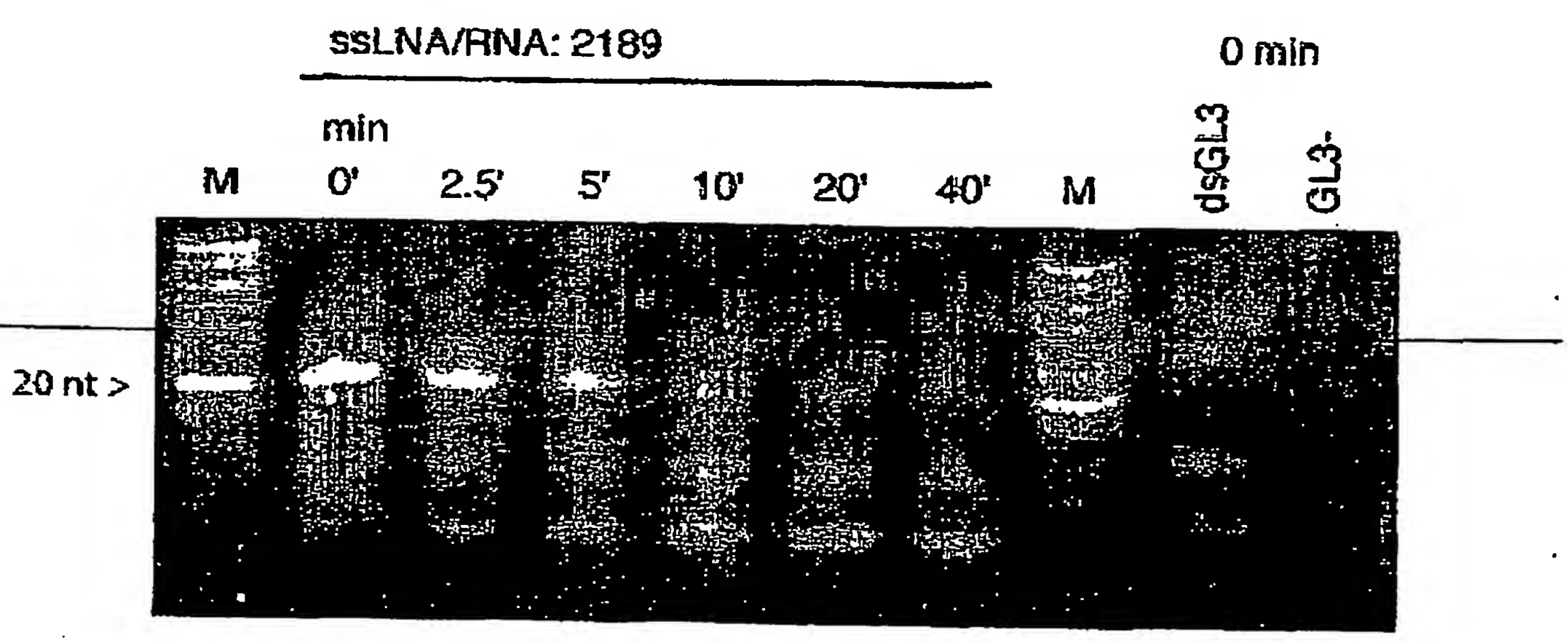

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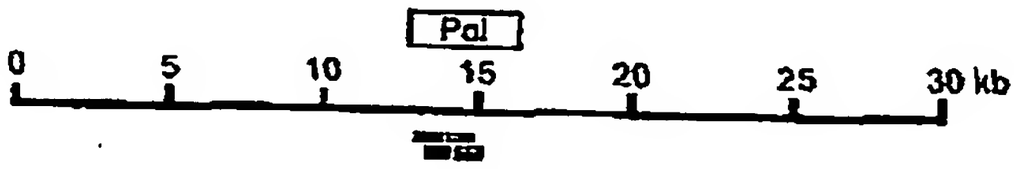
Figure 7/10

siRNA/siLNA targeting SARS



		Start position		End position
siRNA	siRNA SARS 1:	13,547	5'-cgaugaggaaggcaauuuatt-3' 3'-ttccuacuccuuccguuaaau-5'	13,567
	siRNA SARS 2:	14,015	5'-cugguacgauuucggugautt-3' 3'-ttgaccaugcuaaagccacua-5'	14,035
	siRNA SARS 3:	14,595	5'-acugucaaacccgguaauutt-3' 3'-ttugacaguuugggccauuaa-5'	14,615
	siRNA SARS 4:	14,770	5'-gacaacuccuauucguagutt-3' 3'-ttcuguugaggauaagcauca-5'	14,790
siLNA	siLNA SARS 1:	13,547	5'-Cgaugaggaaggcaauuuatt-3' 3'-TTccuacuccuuccguuaaau-5'	13,567
	siLNA SARS 2:	14,015	5'-Cugguacgauuucggugautt-3' 3'-TTgaccaugcuaaagccacua-5'	14,035
	siLNA SARS 3:	14,595	5'-Acugucaaacccgguaauutt-3' 3'-TTugacaguuugggccauuaa-5'	14,615
	siLNA SARS 4:	14,770	5'-Gacaacuccuauucguagutt-3' 3'-TTcuguugaggauaagcauca-5'	14,790

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Figure 8/10

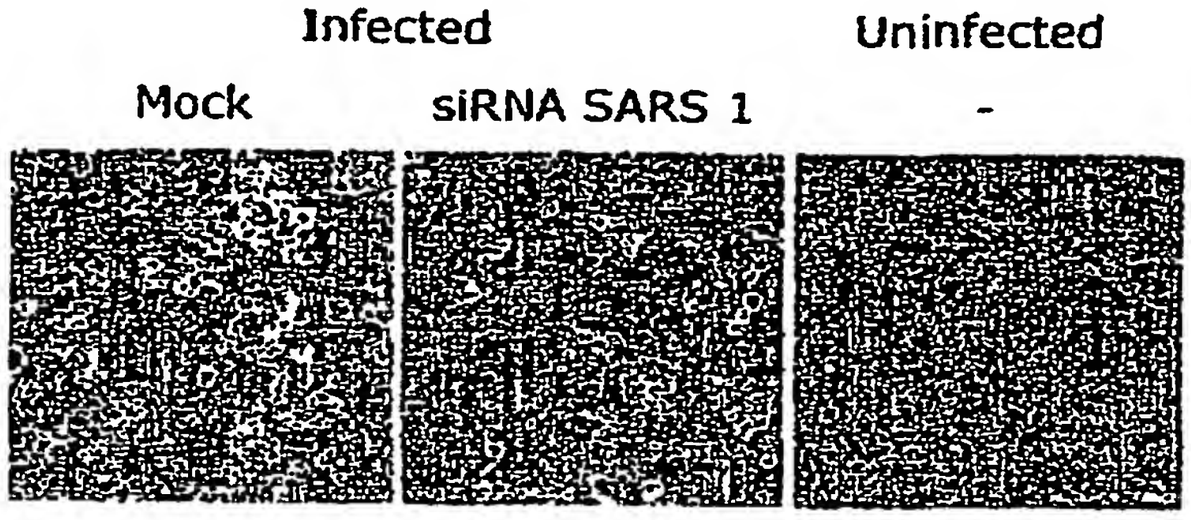

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Figure 9/10

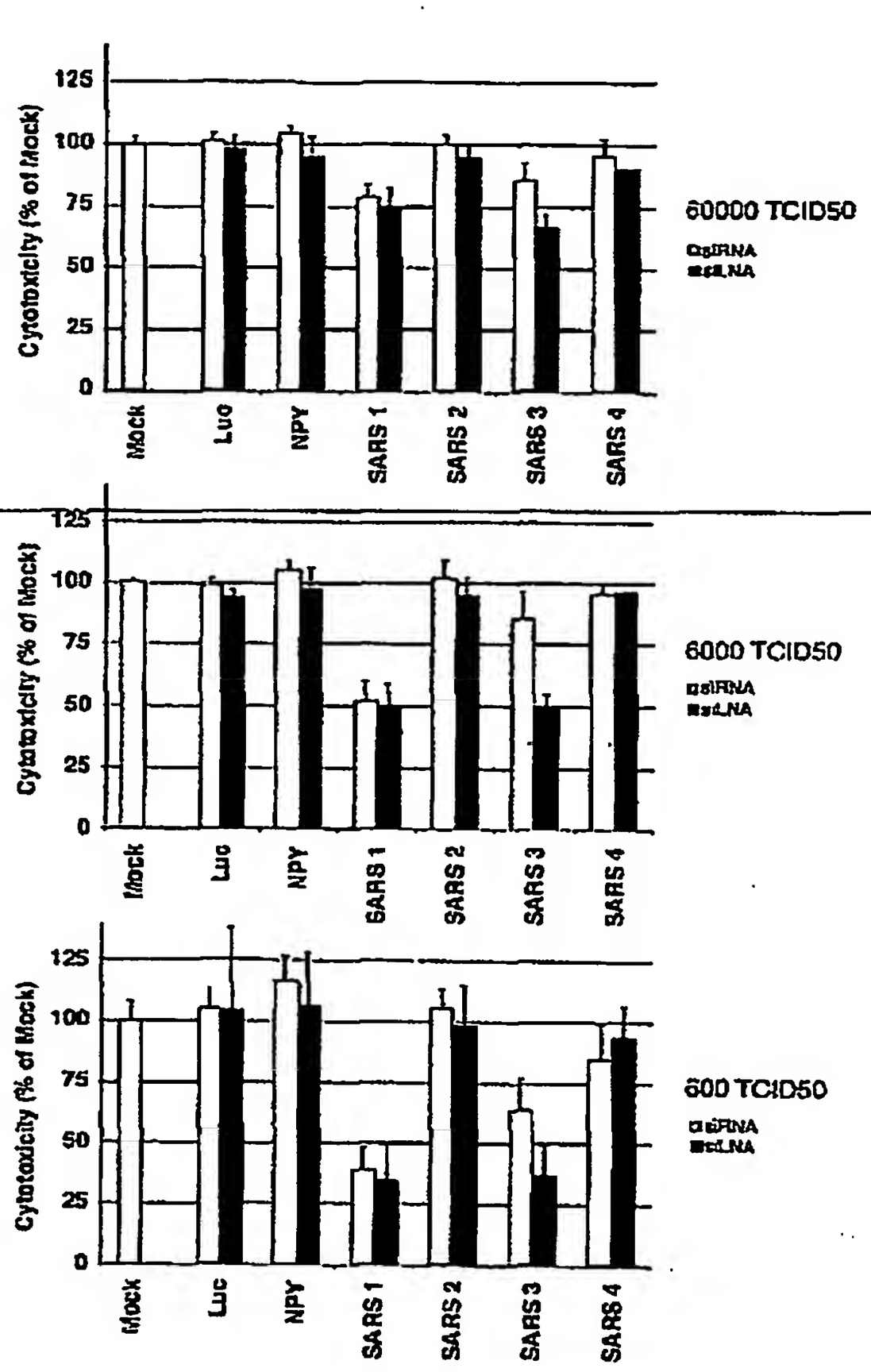

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Figure 10/10